Enzymatic hydrolysis of β-casein and β-lactoglobulin

Foam and emulsion properties of peptides in relation to their molecular structure

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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Stellingen

1. Er bestaan duidelijke relaties tussen de primaire structuur van peptiden en hun schuim- en emulsievormende en -stabiliserende eigenschappen. *Dit proefschrift.*

2. Er bestaat geen rechtstreeks verband tussen de secundaire structuur van eiwitten en peptiden en de in stelling 1 genoemde functionele eigenschappen. *Dit proefschrift.*

3. De hydrolysegraad van een eiwithydrolysaat is een slechte indicator voor structuur-functie relaties.

4. Het voorvoegsel 're-' in de term recoalescentie impliceert dat het proces plaatsvindt na coalescentie en is derhalve geen juiste term voor een emulsievormingsproces.

5. Kennis van structuur-functie relaties is essentieel om levensmiddelen-technologische processen te begrijpen, maar kan (nog) onvoldoende gebruikt worden om deze processen te voorspellen.

6. Aangezien gezuiverde eiwitten zich buiten hun oorspronkelijke milieu bevinden, kunnen ze nooit natief zijn.

7. De trend om wetenschappelijk onderzoek waarvan de toepassing niet direct duidelijk is als geldverspilling te beschouwen, getuigt van een weinig vooruitziende blik.

8. In vele gevallen hebben deeltijdwerkers procentueel meer efficiënte arbeidsuren dan mensen met een volledige weektaak.

9. Vrije verkoop van 'functional foods' kan de gezondheid van de consument ook negatief beïnvloeden.

10. De verschillende dialecten en accenten dragen bij aan de veelzijdigheid van de Nederlandse taal en zijn daarom waardevol.

11. De privatisering van de Nederlandse Spoorwegen heeft gezorgd voor een ontsporing van de prijs/kwaliteit verhouding.

12. Zwangere vrouwen hebben meer plek om een carrière op hun buik te schrijven.

13. In plaats van 'voorkomen is beter dan genezen' lijkt voor Schiphol te gelden: 'gedogen is beter dan beslissen'.

Stellingen behorende bij het proefschrift
'Enzymatic hydrolysis of β-casein and β-lactoglobulin
foam and emulsion properties of peptides in relation to their molecular structure'
Petra W.J.R. Caessens, Wageningen, 9 februari 1999

Abstract

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Keywords: β-casein, β-lactoglobulin, enzymatic hydrolysis, peptides, plasmin, foam, emulsion, mass spectrometry, circular dichroism, structure-function relationship.

Peptides derived from β-casein (βCN) and β-lactoglobulin (βLg) were analysed for their foam- and emulsion-forming and -stabilising properties (further denoted functional properties) and for their structural characteristics in order to elucidate structure-function relationships.

βCN was hydrolysed by plasmin and subsequent fractionation of the hydrolysate resulted in various hydrophilic, amphipathic and hydrophobic peptide fractions with clear differences in functional properties. The highly-charged N-terminal part of the amphipathic peptides appeared to be important for the emulsion-stabilising properties of βCN peptides. The main secondary structure element of βCN(-peptides) in solution was the unordered random coil, but upon adsorption onto an hydrophobic interface α-helix was induced. The hydrophobic C-terminal part of βCN accounted for the high maximum surface load on the interface, while the N-terminal part of βCN seemed to be responsible for the α-helix induction upon adsorption. No clear relation between the secondary structure and the functionality was observed in this system but a relation between a high surface load and good stabilising properties seemed to exist.

Bovine βLg was hydrolysed by the action of trypsin, plasmin and *Staphylococcus aureus* V8 protease. Overall, the plasmin hydrolysate had the best functional properties at pH 6.7, compared to the other hydrolysates and was investigated further. During βLg/plasmin hydrolysis significant SH/SS-exchange has taken place yielding a large number of different peptides. The peptides present were (1) peptides composed of a single amino acid chain lacking a cysteine residue, (2) peptides composed of a single amino acid chain containing intramolecular disulphide bonds and (3) peptides composed of 2 amino acid chains linked by an intermolecular disulphide bond. The occurrence of the SH/SS exchange and the homogeneous distribution of charge and hydrophobicity hinder an efficient fractionation of the hydrolysate.

In conclusion, the production of specific peptides and peptide fractions is more complicated for βLg than for βCN, mainly because of the differences in primary structure (such as the distribution of charge and hydrophobicity) between the proteins. The foam- and emulsion-forming properties of peptides can be superior to those of intact proteins, as long as they have both charged and hydrophobic areas. The foam- and emulsion-stabilising properties of peptides depend highly on the amount of repulsion they can produce (either by a strong amphipathicity or by a high surface load).

aan mijn ouders

Contents

Chapter 1

General introduction

Background

Already in 1974 the influence of proteolysis of whey protein concentrate (WPC) on its functional properties was investigated (Kuehler and Stine, 1974). Several studies on the foam, emulsifying and interfacial properties of milk protein hydrolysates have been published since then. Often, the functional properties of mixtures of proteins (i.e. whey proteins or caseinates) were investigated and because pure systems were not used, it is difficult to deduce a structure-function relationship. Another problem in this research area is that many different methods have been used to investigate the foam and emulsion properties. This makes it difficult to compare the results from these investigations (Halling, 1981; Sherman, 1995). Consequently, most of the understanding concerning the foam and emulsifying properties of hydrolysates for utilisation in the food industry is based on empirical knowledge. The present study was initiated with the aim of elucidating different aspects that are important for the structure-function relationship of proteins and protein hydrolysates with respect to their foam- and emulsion-forming and -stabilising properties. The proteins used were β-casein and β-lactoglobulin, two structurally different, well-characterised milk proteins.

β-Casein and β-lactoglobulin

Bovine milk contains roughly 30-35 g of protein g per L. About 80% of these proteins consist of the caseins, a group of proteins with little organised secondary structure that occur in micellar form. Casein is divided into 4 groups of proteins: α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. The other 20% of the total milk protein consist of the whey proteins (also called milk serum proteins), which include α-lactalbumin, β-lactoglobulin, immunoglobulins, serum albumin, and several minor proteins (Swaisgood, 1982; Walstra and Jenness, 1984).

β-Casein

Approximately 35% of the caseins are β-casein (βCN), a protein with a monomer molecular mass of approximately 24 kDa. Currently, eight different genetic variants of βCN are known, called A^1, A^2, A^3, B, C, D, E (Swaisgood, 1992) and F (Visser et al., 1995); in Western breeds of cattle, the A^1 and A^2 variants occur with the highest frequency.

In fresh milk, βCN can be degraded by plasmin (naturally present in milk) into several large degradation products, the γ-caseins (γ_1, γ_2 and γ_3), and the complementary parts known as the proteose peptone fractions 5, 8F and 8S (Andrews, 1978a, b; Eigel et al., 1984).

Figure 1 shows the primary structure of βCN. The protein contains five phosphoserine residues, all located in the N-terminal part of the molecule (residues 15, 17, 18, 19, 35). At pH 6.6, the N-terminal fragment of 21 residues carries a net charge of -12, whereas the remainder of the molecule has no net charge. The distinct separation of the N-terminal part containing many charged groups, and the C-terminal part containing many hydrophobic and only little charged groups, makes βCN an amphipathic protein, having detergent-like properties. At temperatures below 4°C, βCN exists as a monomer (Payens and Van Markwijk, 1963), whereas at higher temperatures and above a critical concentration micelles are formed (Schmidt and Payens, 1972). Furthermore, βCN contains a high amount of proline residues (~17%), which might be the reason why the molecule has only a low amount of ordered secondary structure (Creamer et al., 1981; Graham et al., 1984; Swaisgood, 1992).

1
H. Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser^{P}-Leu-Ser^{P}-Ser^{P}-Ser^{P}-Glu-

21
Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-Ser^{P}-Glu-Glu-Gln-Gln-Gln-

41
Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-

61
Pro-Phe-Pro-Gly-Pro-Ile-His$^{A^1}$-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-
Pro$^{A^2}$
81
Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-

101
Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr-

121
Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-

141
Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-

161
Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-

181
Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-

201
Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val.OH

Figure 1 Primary structure of βCN A^1 and A^2 (Swaisgood, 1992); the phosphate groups are indicated with ^{P}.

β-Lactoglobulin

β-Lactoglobulin (βLg) is the major whey protein, representing about 50% of all whey proteins. At this time eight genetic variants are known in Western breeds, called A, B, C, D, H, I, J and W (Creamer and Harris, 1997), of which the variants A and B are most often present in commercial preparations (Hambling et al., 1992). Bovine βLg has a monomer molecular mass of about 18.3 kDa. The primary structure of βLg is shown in Figure 2. βLg is a globular protein containing five cysteine residues, of which four are involved in disulphide bonds. One disulphide bond is between residues 66 and 160, the other between residues 106 and 119, whereas residue 121 contains a free thiol group. It has also been suggested that the free thiol is equally distributed between the residues 119 and 121 (Swaisgood, 1982; Hambling et al., 1992).

Figure 2 Primary structure of βLg A and B (Hambling et al., 1992); the lines show disulphide bonds; * shows the free thiol group.

In milk (more generally, between pH 5.2 and 7.5) native βLg occurs as a dimer. The association behaviour of the protein depends on both pH and temperature (Kinsella and Whitehead, 1989). Below pH 3.5 βLg dissociates into its monomers (Townend et al., 1960a), whereas between pH 3.5 and 5.2 it reversibly forms tetramers/octamers (Townend et al., 1960b; Townend and Timasheff, 1960). Above

pH 7.5, βLg starts to unfold, with a concomitant increase in the reactivity of the free thiol group (Tanford et al., 1959). At temperatures between 30 and 55°C, the native βLg dimer also dissociates into monomers and at even higher temperatures the molecule unfolds, resulting in an increased reactivity of the free thiol group (Hambling et al., 1992). Native βLg is resistant to gastric digestion (Miranda and Pelissier, 1983) and to peptic and chymotryptic hydrolysis, because of its stable conformation (Reddy et al., 1988). The biological function of the protein remains unclear but there are strong indications that it has a transport function in the intestinal tract of the neonate (Papiz et al., 1986; Hambling et al., 1992).

Enzymatic hydrolysis

Functional properties of proteins can be modified in several ways, e.g. by physical, chemical, enzymatic or genetic modification (Whitaker and Puigserver, 1982; Phillips et al., 1994). Enzymatic modification has a long tradition in the food industry, for instance in cheese production, the baking industry, the beer industry, etc. and it is a well-accepted means of protein modification (Whitaker and Puigserver, 1982; Adler-Nissen, 1986; Visser et al., 1993). During enzymatic hydrolysis, the peptide bonds in a protein are cleaved by a catalytic action of a protease. Given the specificity of a protease for particular peptide bonds, proteins can have a preference for certain of these peptide bonds within a protein (Visser, 1981; Adler-Nissen, 1986). The degree of hydrolysis (DH) is a measure of the extent of cleavage of peptide linkages, and can be calculated as follows:

$$\%DH = (h / h_{tot}) \times 100\%$$

with h being the hydrolysis equivalents in meqv/g protein, and h_{tot} being the total number of peptide bonds in the protein substrate in meqv/g protein. An intact protein has a DH of 0% (i.e. all peptide bonds are intact), and a protein that is completely degraded into amino acids has a DH of 100% (i.e. all peptide bonds are split; Adler-Nissen, 1986).

Enzymatic hydrolysis of proteins can have several purposes, such as to prepare microbiological media, feed or food supplements and to reduce allergenicity. During protein hydrolysis, bitter-tasting peptides can be yielded, which is often unwanted (Adler-Nissen, 1986). In this study, enzymatic hydrolysis was used as a tool to modify proteins, with the aim of altering the foam- and emulsion-forming and -stabilising properties. Enzymatic hydrolysis of proteins results in a decreased molecular mass, an increased amount of ionizable groups and the exposure of hydrophobic groups (Lahl and Braun, 1994; Panyam and Kilara, 1996). Functional properties of proteins, such as solubility and surface activity, are altered by this treatment. Consequently, enzymatic hydrolysis has an impact on the ability of proteins to form and stabilise foam and emulsions.

Formation and stabilisation of foam and emulsions

Foam and emulsions are systems in which one phase (air for foam and oil for oil-in-water emulsions) is dispersed in another phase. Although foam and emulsions are both dispersed systems and the processes that take place in the formation and stabilisation are similar, there are also several differences between the systems. These include the particle diameter (i.e. in the order of 0.1-1 mm and 0.2-10 μm, respectively), the solubility of the dispersed phase in the continuous phase, the interfacial tension, and others (Walstra, 1987; Prins, 1988).

It is important to discriminate between the formation and the stabilisation of foam and emulsions, since different aspects are important. The formation of a foam or emulsion requires a surfactant to lower the surface tension and energy input to break up the air and oil into bubbles and droplets. The rupture of a bubble or droplet into smaller ones is difficult because of the Laplace pressure (p_L), given by

$$p_L = 2\gamma/R$$

with γ being the surface tension and R being the radius of the particle. Various aspects are important during the formation, such as the adsorption of the surfactant, breaking up the dispersed phase and the (in)stability of the newly formed particles during the formation; all these phenomena occur simultaneously (Walstra, 1993). Surfactants not only play a role in the formation of foam and emulsions but also in preventing the bubbles and droplets from becoming unstable. Foams and emulsions are exposed to different instability processes as shown in Figure 3. Creaming and drainage are caused by a density difference between the phases. Disproportionation (also called Ostwald ripening) can occur when the dispersed phase is soluble in the continuous phase (consequently, this will take place in foams but not in emulsions formed with a triglyceride oil). Aggregation (or flocculation) is the process whereby particles stick together. Coalescence occurs when the film between two particles breaks so that they flow together. Partial coalescence may occur in emulsions with fat crystals present (Walstra, 1996).

The role of proteins in foam and emulsion formation and stabilisation

In many industrial products, proteins are used for their foam- and emulsion-forming and -stabilising properties (further also denoted functional properties) since they can adsorb at oil/water and air/water interfaces, and thereby lower the surface tension. In contrast to the conformation of a low molecular mass surfactant, that of proteins might change upon adsorption, since the hydrophobic side chains favour the oil phase, and the hydrophilic residues prefer the aqueous phase (Dickinson, 1994; Horne, 1996; Dalgleish, 1997). When a protein (or more generally, a surfactant) is adsorbed at an interface, it changes the rheological properties of the interface. This has consequences for the foam- and emulsion-stabilising properties of the protein, because it can influence drainage of the liquid layer between the bubbles or droplets (Halling, 1981; Prins, 1990; Walstra and De Roos, 1993). Furthermore, proteins can stabilise by electric repulsion, which is influenced by for

instance pH and ionic strength, and by steric repulsion, which depends on the protein conformation and on the packing behaviour or surface-load of the protein (Dalgleish, 1997).

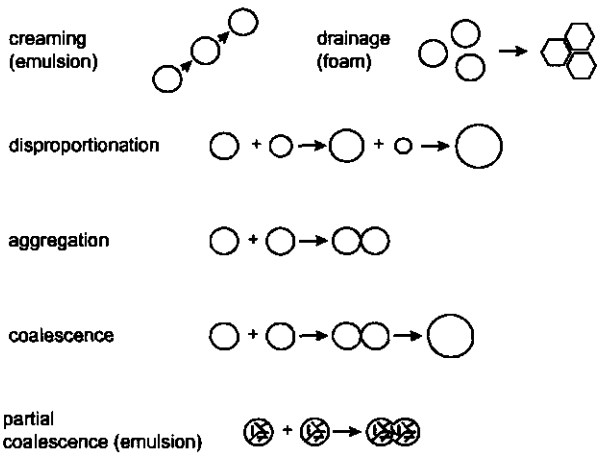

Figure 3 Types of instability in foam and oil-in-water emulsions

The functional properties of milk proteins have been well studied (reviews on this subject are: Kinsella and Whitehead, 1989; Mulvihill and Fox, 1989; De Wit, 1989; Morr and Ha, 1993; Phillips et al., 1994; Wong et al., 1996). Several factors, such as molecular size, hydrophobicity, charge and flexibility were reported as important for the functional properties of proteins. Of course, external factors, such as temperature, pH, ionic strength and the presence of other molecules (e.g. lipids or carbohydrates) will influence these functional properties as well (Phillips et al., 1994).

The difference in structural properties between βCN and βLg (having a typically random-coil and globular character, respectively) is mentioned as the reason for their differences in functionality (Mulvihill and Fox, 1989). βCN is thought to adsorb onto oil/water and air/water interfaces in a loop-and-train conformation, whereas a globular protein like βLg has a more rigid structure, which can unfold in the adsorbed state (Walstra and De Roos, 1993; Dickinson, 1994); Figure 4 shows a schematic picture of these proteins in the adsorbed state. Lorient et al. (1989) showed that of the caseins, βCN is the most efficient in lowering the surface tension, and possesses the highest foaming capacity; it is also a well-known emulsifier (Dickinson, 1994). The surface activity and the emulsifying properties of βLg are highly dependent on the pH (Klemaszewski et al., 1992; Wong et al., 1996). Of the whey proteins, βLg possesses the best foam-forming properties (De Wit, 1989).

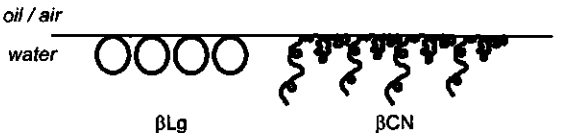

Figure 4 Possible conformation of native βLg and βCN when adsorbed at an air/water or oil/water interface; highly schematic and not to scale.

Functional properties of milk protein hydrolysates/peptides

Many papers on the foam, emulsifying and interfacial properties of milk protein hydrolysates have been published. Without being complete, an overview of studies on this subject is given in Table 1. It is difficult to deduce structure-function relationships, due to the different methods that have been used to investigate foam and emulsions. Another problem is the presence of mixtures of proteins and/or peptides in the systems investigated (Table 1). An additional example of this is that in a combination of whey proteins and amphipathic peptides (formed by the action of endogenous proteases present in whey) these peptides influenced the functionality of the proteins primarily by affecting their surface activity and colloidal properties (Haque, 1993).

In several studies mentioned in Table 1, the peptide composition was characterised and the identification of some peptides was given, which is helpful in understanding structure-function relationships. For instance, Turgeon et al. (1992) reported that the peptide fraction from βLg with increased interfacial properties contained the peptides βLg[f 21-40] and βLg[f 41-60], whereas the fraction containing the peptides βLg[f 61-69] -S-S- [f 149-162] and βLg[f 61-70] -S-S- [f 149-162] had poor interfacial properties. The uniform distribution of charge and hydrophobicity, as well as the rigid structure due to the disulphide bond, were mentioned as reasons for the poor functionality of the fraction containing the latter peptides (Turgeon et al., 1992). Huang et al. (1996) reported the preparation of a peptide fraction from βLg that had improved emulsifying properties. The major peptide present had a molecular mass of 8.4 kDa, and was identified as βLg[f 41-100] -S-S- [f 149-162]. In that study it was shown that the emulsifying property of this peptide was superior to that of βLg[f 41-60] (described by Turgeon et al., 1992).

Other investigations focus on more specific peptides instead of peptide mixtures. For instance, Shimizu et al. (1984) found that α_{s1}-casein [f 1-23] had a similar emulsifying activity to that of the intact molecule (at concentrations higher than 2% w/v). However, in a later paper of the same authors (Shimizu et al., 1986), it was concluded that the emulsifying activity of the purified α_{s1}-casein [f 1-23] arose from some kind of synergistic effect with other peptides present in the crude α_{s1}-casein [f 1-23] fraction. In another study (Lee et al., 1987a), it was shown that both the

Table 1 Short literature overview on functional properties of milk protein hydrolysates

Reference	Substrate	Enzyme used	Conditions of hydrolysis and possible additional treatment	Main results and/or conclusions (hydrolysate compared to intact protein)
Chobert et al. (1988a)	total casein	*Staphylococcus aureus* V8 protease	pH 7.8; 45°C; DH 2% and 6.7%	increased solubility around iso-electric pH; decreased emulsifying activity
Chobert et al. (1988b)	total casein	trypsin	pH 8.0; 37°C; DH 4.3%, 8.0% and 9.9%	increased solubility around iso-electric pH; increased emulsifying capacity and activity
Haque & Mozaffar (1992)	total casein	trypsin, chymotrypsin, rhozyme-41	pH 8.0; 4°C; DH between 2% and 5%	increased solubility and emulsifying properties of all hydrolysates (rhozyme-41 formed hydrolysate with best emulsifying activity; trypsin formed hydrolysate with best emulsion stabililty)
Patel (1994)	total casein	alcalase, chymotrypsin, neutrase, trypsin, pepsin, acid fungal protease, prozyme, pronase E.	pH/temperature 8.5/50°C, 7.8/25°C, 7.5/50°C, 7.6/25°C, 3.5/37°C, 5.0/30°C, 5.0/30°C and 7.537°C for several enzymes respectively; DH 5% and 10% ultrafiltration (MWCO 10 kDa)	all hydrolysates increased solubility around iso-electric pH; generally permeates higher foam capacity than retentates; foam stability decreased, except for the DH5%-retentate formed by pepsin and DH5%-permeate formed by acid fungal protease
Agboola & Dalgleish (1996)	total casein	trypsin	E/S = 1/1000 (hydrolysis of protein in solution) and E/S = 1/2000 (hydrolysis of protein adsorbed on emulsion droplet); pH 7; 25°C; different times	decreased emulsion stability with protein hydrolysates; marginally improved emulsion stability after hydrolysis of adsorbed proteins
Slattery & Fitzgerald (1998)	total casein	*Bacillus* protease	pH 7.5; 50°C; DH 0.5%, 1.0%, 3.0%, 9.0% and 15.0%	DH 0.5% and 1.0% had increased emulsifying (pH 2 and 4) and foaming (pH 8 and 10) properties; decreased emulsifying properties at higher DH
Chobert et al. (1989)	βCN	trypsin	pH 8.0; 37°C; DH 3.2%, 5.0%, 5.8% and 7.4%	increased solubility (pH 4.0-7.5) and emulsifying activity (pH 1.5-3.5 and pH 6.5-10.0); decreased emulsion stability
Kuehler & Stine (1974)	WPC	pepsin, pronase, prolase	pH 2.5 (pepsin) and 7.5 (pronase, prolase); 50°C; different times	decreased emulsifying properties; optimal hydrolysis time for increased foam-forming properties; decreased foam stability

Jost & Monti (1982)	WPC	trypsin, chymotrypsin, pepsin, papain, subtilisin, thermolysin, thermitase, pancreatin, *Bacillus licheniformis* protease, *Bacillus subtilis* protease	not mentioned ultrafiltration (to remove fat and phopholipids)	positive correlation between surface activity and peptide chain length (minimum length of 20 residues necessary for functionality); only the trypsin hydrolysate had improved emulsifying properties; this effect was pH-dependent (best result at pH > 7)
Chobert et al. (1988b)	WPC	trypsin	pH 8.0; 37°C; DH 2.5%, 3.9 and 5.3%	increased solubility around iso-electric pH; *increased emulsifying capacity and -activity*
Turgeon et al. (1991) Gauthier et al. (1993)	WPC (normal and heat treated)	trypsin, chymotrypsin	E/S = 1/200; pH 8; 40°C; 45 min Two-step ultrafiltration (MWCO 30 and 1 kDa)	trypsin treatment: increased interfacial properties (pH 4); relation with changed emulsifying capacity suggested; optimum mean molecular size for improved functionality
Althouse et al. (1995)	WPI	acid fungal protease, alcalase, trypsin, pepsin, *chymotrypsin*,	pH/temperature 5.0/30°C, 8.5/30°C; 7.6/25°C, 2.0/37°C and 7.8/25°C for *several enzymes respectively*; DH 2.5-3%; ultrafiltration (MWCO 10 kDa)	increased foam-forming properties; permeate of WPI/alcalase hydrolysate had *the best foaming characteristics*
Lieske & Konrad (1996)	WPC	papain	E/S = 1/50; pH 6.5; 48°C; several times thermal unfolding at acid pH	optimal DH for functionality was 3% characterised by a reduction of aggregated whey protein; liberated peptides supported foaming but not emulsification
Turgeon et al. (1992)	βLg	trypsin	E/S = 1/200; pH 8; 40°C; 45 min Two-step ultrafiltration (MWCO 30 and 1 kDa)	some fractions had improved interfacial properties
Huang et al. (1996)	βLg	trypsin	hydrolysis in immobilised-trypsin bioreactor, with immediate membrane fractionation (MWCO 3 kDa) of limited hydrolysate	slightly improved interfacial tension; largely improved emulsifying properties
Agboola & Dalgleish (1996)	βLg	trypsin	E/S = 1/25 (hydrolysis of protein in solution) and E/S = 1/1000 (hydrolysis of protein adsorbed on emulsion droplet); pH 7, 25°C different times	decreased emulsion stability with protein hydrolysates; marginally improved emulsion stability after hydrolysis of adsorbed proteins

Abbreviations used:

βCN β-casein
βLg β-lactoglobulin
DH degree of hydrolysis
E/S enzyme /- substrate ratio
MWCO molecular weight cut off
WPC whey protein concentrate
WPI whey protein isolate

hydrophobic peptide βCN[f 193-209] and the hydrophilic peptide βCN[f 1-25] had a low emulsifying activity at neutral pH. In both the acidic and alkaline pH region the emulsifying activity of the hydrophobic peptide was high; at acidic pH the hydrophilic peptide also had a high emulsifying activity. Because at pH 3 both peptides were more surface-active than at pH 7, a relation with the surface activity was suggested. In a later paper of the same authors (Lee et al., 1987b), a synergistic effect between the hydrophobic peptide βCN[f 193-209] and the hydrophilic glycomacropeptide derived from κ-casein was reported, which resulted in an improved emulsifying activity.

Although it is difficult to compare the different results found for milk protein hydrolysates (for reasons mentioned above), some general conclusions can be drawn. Firstly, the enzyme specificity is important for the functionality of the hydrolysates (although different authors mentioned different enzymes to be beneficial; Table 1). Secondly, a limited degree of hydrolysis could improve the functional properties and in order to maintain good functional properties, the apparent molecular mass of the peptides should not be lower than approximately 2 or 5 kDa, as mentioned by Turgeon et al. (1992) and Chobert et al. (1988b), respectively. Thirdly, the influence of hydrolysis on the functionality appeared to be larger for whey proteins than for casein; this is probably because the hydrophobic residues buried in the globular whey proteins are able to interact with the oil-phase after hydrolysis (Chobert et al., 1988b). Fourthly, it was shown that the presence of minor amounts of other peptides can have a great impact on the functional properties of peptides (Shimizu et al., 1986; Lee et al., 1987b). Finally, it has been reported that characteristics for good interfacial properties are clustering of hydrophilic and hydrophobic residues in distinct zones and a minimum molecular mass of the peptide allowing this distribution (Turgeon et al., 1992).

Purpose of the study

Despite the number of studies concerning the functional properties of milk protein hydrolysates, the structure-function relationship of the proteins and their foam- and emulsion-forming and -stabilising properties has remained unclear. A large, collaborative project, called "Formation and stabilisation of emulsions and foam with proteins and peptides in relation to their molecular properties", was initiated to elucidate the structure-function relationship of proteins in regard to the functional properties mentioned. The overall project was divided into three sub-projects: foam, emulsions, and enzymatic hydrolysis. The first two sub-projects, not covered in this thesis, were focused on the physical aspects of foam and emulsions that were formed and stabilised by proteins and peptides (Van Kalsbeek, 1999; Smulders, 1999). The third sub-project, which is described in this thesis, dealt with

the enzymatic hydrolysis of proteins in order to modify their foam- and emulsion-forming and -stabilising properties.

The goal of the present study was to elucidate the structure-function relationship for peptides from βCN and βLg, two structurally different, well-characterised milk proteins, by using a systematic approach. Therefore, the proteins were modified by enzymatic hydrolysis, the reaction products were fractionated and characterised and the foam- and emulsion-forming and -stabilising properties of the characterised peptide mixtures and peptides were determined. The results enabled the discussion of structure-function relationships of the peptides on a molecular level.

Outline of the thesis

Chapters 2, 3 and 4 describe the investigation of the structure-function relationship of βCN peptides with respect to their foam- and emulsion-forming and -stabilising properties. In Chapter 2, the plasmin hydrolysis of βCN and the fractionation of the hydrolysate is described. Foam- and emulsion-forming and -stabilising properties and surface-active properties of the peptide fractions are discussed in relation to the peptide composition of the several fractions. In Chapter 3, the identification of the βCN peptides and the purification of the peptides responsible for emulsion-stabilising properties is described. A hypothesis for the structure-function relationship, based on the primary structure of the peptides, is postulated. An investigation of the relation between secondary structure of βCN (-peptides) and foam- and emulsion-forming and -stabilising properties is described in Chapter 4.

Chapters 5, 6 and 7 concern the structure-function relationship of βLg peptides. The purification and physico-chemical characterisation of native βLg is described in Chapter 5. Chapter 6 describes the influence of βLg hydrolysis by plasmin, trypsin and *Staphylococcus aureus* V8 protease on the foam- and emulsion-forming and -stabilising properties. In Chapter 7, the fractionation of a βLg/plasmin hydrolysate and the identification of the βLg peptides are described. Functional properties of the peptide fractions are discussed in relation to the peptide composition.

Finally, Chapter 8 gives a general discussion of the total thesis work.

Chapter 2

Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate

Abstract

Bovine β-casein (βCN) was hydrolysed by plasmin. The hydrolysate was fractionated by ultrafiltration and selective precipitation, which resulted in several peptide fractions of which the peptide composition was monitored by reversed-phase HPLC.

Poorly soluble, hydrophobic peptide fractions, containing peptides from the C-terminal half of the βCN sequence, possessed improved foam-forming and -stabilising properties compared to those of intact βCN, especially at pH 4.0. Soluble peptide fractions, containing a variety of peptides from the "middle" part of the βCN sequence in different proportions, possessed improved emulsion-forming capacity at pH 6.7, compared to intact βCN, and showed large variations in emulsion stability. The fraction containing the hydrophilic N-terminal part of βCN showed inferior foam, emulsion and surface-active properties, especially at pH 6.7.

The differences in functionality found between the various peptide fractions may either be attributed to synergistic effects between peptides or to a specific functionality of some individual peptides.

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Introduction

The strongly amphipathic nature of proteins enables them to adsorb onto interfaces, and this functionality is exploited by food and pharmaceutical industries to stabilise foams and emulsions (Mulvihill and Fox, 1989). The functionality of proteins can be altered by chemical, physical, enzymatic, and genetic modification (Phillips et al., 1994). Enzymatic modifications have the advantage of mild reaction conditions (Arai and Fujimaki, 1991; Phillips et al., 1994), and can be used to enhance some functionalities of food proteins (Panyam and Kilara, 1996). Enzymatic hydrolysis of proteins has several consequences: decrease in molecular weight (MW); increase in the number of ionizable groups; exposure of hydrophobic groups; etc. (Panyam and Kilara, 1996). This implies that the solubility and the surface activity of the material will be altered, and, consequently, the propensity of the hydrolysed protein to form and stabilise foams and emulsions may be different from that of the intact protein.

Plasmin is a proteolytic enzyme with a preference for Lys-X and Arg-X bonds. In milk the enzyme is associated with casein micelles in which α_{s2}- and β-casein (βCN) are the most susceptible to plasmin degradation (for a review see Bastian and Brown, 1996). In fresh milk βCN is converted by plasmin into several large, intermediate degradation products, the γCNs, and to the complementary parts called proteose peptones (Andrews, 1978a, b; Eigel et al., 1984). Wilson et al. (1989) modified isolated βCN by plasmin hydrolysis to yield γ-caseins and proteose peptones. The surface pressure at the air/water (A/W) interface induced by these hydrolysis products appeared to be different from that of the intact protein. Considering the expected relation between surface-active properties and foam and emulsion properties (Mulvihill and Fox, 1989), it can be expected that the foam and emulsion properties of βCN are altered by plasmin hydrolysis.

Shimizu et al. (1984) found that the α_{s1}CN[f 1-23] peptide fraction showed an emulsion-activity index similar to intact α_{s1}CN at concentrations higher than 2% (w/v). However, in a later paper of the same authors (Shimizu et al., 1986), a synergistic effect in emulsion activity between the studied α_{s1}CN[f 1-23] and traces of coexisting peptides was demonstrated. Such a synergistic effect was also mentioned by Lee et al. (1987b). The objective of the study presented in this Chapter was to produce, by means of plasmin hydrolysis, well-defined peptide mixtures from βCN with altered foam, emulsion, and surface-active properties. To that end, we fractionated the hydrolysate stepwise to produce special peptide fractions, so that the influence of removing certain peptides could be tested.

Materials and Methods

Materials

Bovine βCN (90% βCN based on dry weight, 95% βCN based on 100% nitrogen, w/w) was purchased from Eurial (France). Bovine plasmin (EC 3.4.21.7) and aprotinin

were obtained from Sigma (USA; article numbers P-7911 and A-6012, respectively). Unless stated otherwise, all other chemicals were of analytical grade, and were purchased from Merck (Germany) or BDH (UK).

β-Casein hydrolysis and fractionation of the hydrolysate

Figure 1 shows an outline of the βCN hydrolysis and the fractionation of the hydrolysate. Bovine βCN was hydrolysed by plasmin (3% w/v βCN; enzyme/substrate = 1/2300 w/w; pH 6.8; 40°C) using a pH-stat method (Adler-Nissen, 1986). During the hydrolysis a precipitate formed (this precipitate disappeared at temperatures lower than approximately 4°C). The precipitate was removed by centrifugation (1500×*g*, 10 min, 30°C), and the resulting supernatant was incubated yielding further precipitates, which were repeatedly centrifuged off. Centrifugation was after 2, 2.5, 3, 3.75, 4.5, 5.5 and 7 hours of hydrolysis (called: "hydrolysis with intermediate pellet separation"). The pellets obtained from the several centrifugation steps were combined, and the total resulting pellet (PEL-1) was dissolved in the original volume of water, adjusted to pH 10, after which part of it was hydrolysed further with plasmin (enzyme/substrate = 1/2300 w/w; pH 8.0; 40°C; pH-stat method; 5 hours). The hydrolysis was terminated by adding aprotinin to the reaction mixture (ratio 1/200, v/v). The two resulting hydrolysates (the supernatant of the βCN-hydrolysate, SUP-1, and the hydrolysate of

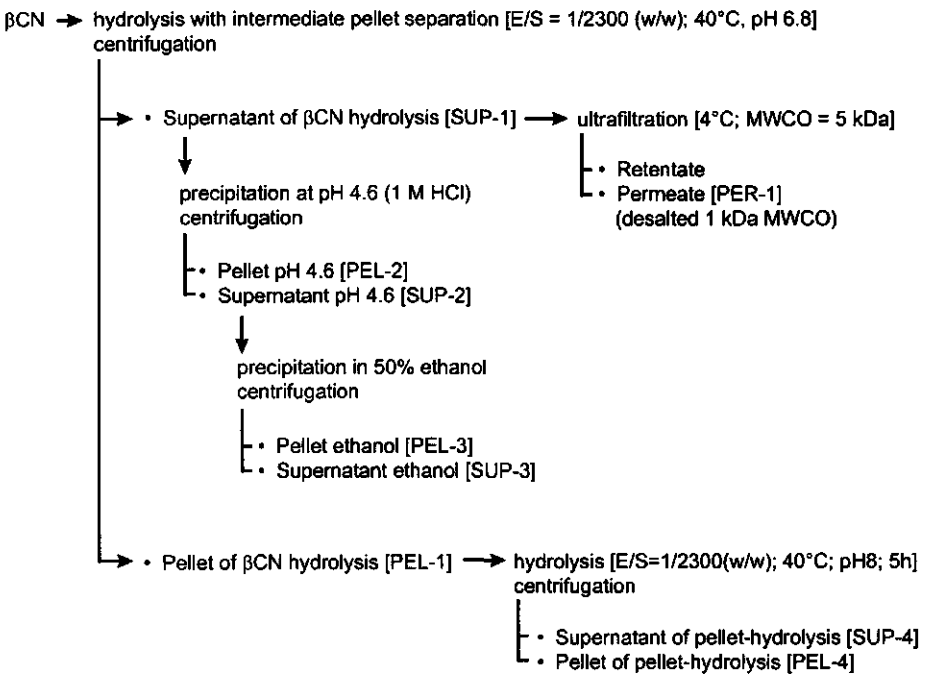

Figure 1. Outline of the βCN hydrolysate fractionation.

PEL-1) were fractionated by means of selective precipitation (SUP-2, SUP-3, PEL-4 and SUP-4) and ultrafiltration (RET-1 and PER-1; Filtron mini-ultra-set, OMEGA™ membrane, MWCO 5 kDa, Pall Filtron Corp., USA) as outlined in Figure 1. Before ultrafiltration (UF), the supernatant of the βCN hydrolysate (SUP-1) was diluted 3-fold with 2 M acetic acid. After 50% UF (50% reduction of the volume), the retentate was diafiltered (DF) with 1.3 M acetic acid (200% DF, which means 2 times the volume of the retentate is used as diawater), after which the retentate was washed with double-distilled water (200% DF) followed by 50% UF. All peptide fractions were lyophilised and stored at 4°C before further analysis.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The peptide composition of the βCN hydrolysate fractions (Figure 1) was analysed by RP-HPLC. The RP-HPLC equipment used is described by Visser et al. (1991). Solvent A (0.1% trifluoroacetic acid [TFA] in 10% aqueous acetonitrile, v/v) and solvent B (0.08% TFA in 90% aqueous acetonitrile, v/v) formed the eluent in the following linear gradient steps: from 10% to 16% B over 2 min followed by 2 min isocratic elution; to 26% B over 8 min; to 35% B over 4 min; to 38% B over 32 min; then to 70% B over 3 min and finishing with 5 min isocratic elution at 70% B. A flow rate of 0.8 mL/min was applied. Generally, 50 μL of a 1 mg.mL protein solution was injected. Peak detection and quantitation was performed at 220 nm using Turbochrom data-acquisition and processing software (Perkin-Elmer, Germany).

Capillary Electrophoresis (CE)

RET-1 and SUP-3 (Figure 1) were analysed by CE, basically following de Jong et al. (1993). The CE equipment used was a Beckman P/ACE System 5500 controlled by Beckman Software Gold System Version 8, using a hydrophilically coated Supelco capillary (USA), 27 cm × 50 μm, and a 5 mM sodium citrate - 6 M urea - 0.05% (w/v) methylhydroxyethyl cellulose (Tylose, Hoechst, Germany) buffer adjusted to pH 2.5 with citric acid. CE-separation was performed at 10 kV and 30°C for 60 min. Peak detection was performed using UV-absorption at 214 nm.

Mass Spectrometry (MS)

Some peptide components from PER-1, SUP-1, SUP-3, and PEL-1 (Figure 1) were collected (semi-preparative RP-HPLC) and identified by electrospray-ionization MS (ESI-MS). This was performed on a VG Platform quadrupole mass spectrometer (Micromass, UK). Samples were dissolved in a mixture of acetonitrile - water - formic acid (500 : 500 : 1, v/v/v) and injected into a flow of acetonitrile - water (1 : 1, v/v, 5 μL/min). The potential at the capillary tip was maintained at 3.2 kV; the cone voltage was 30 V. Nitrogen was used as nebulizing and drying gas. Calibration was performed using horse heart myoglobin. The raw mass spectral data were processed and transformed with the Masslynx software version 2.2 (Micromass, UK). Peptide identification was obtained from the molecular masses determined, combined with sequence data of the protein and the known specificity of plasmin. The maximum

difference between the mass values measured and the theoretical values was approximately 1.5 Da.

Functional Properties ("foam", "emulsion", "surface activity")

Foam, emulsion, and surface-active properties of the peptide fractions were tested in small-scale screening tests at pH 6.7, which is the pH of milk, and I = 0.075 (0.02 M HCl-imidazole buffer containing 3.44 g/L NaCl and 0.2 g/L NaN_3) and at pH 4.0, which is representative for acidic foods, and I = 0.075 (0.03 M citrate buffer containing 3.15 g/L NaCl and 0.2 g/L NaN_3) at 20°C.

Foam-forming and -stabilising ability was tested with a whipping method: a volume of 100 mL of a 0.01% (w/v) protein solution was placed in a graduated glass cylinder and whipped for 70 s at 2500 rpm using a small impeller (Figure 2). Foam height was monitored for 1 hour (the first measurement at 2 min after starting stirring), and the foam quality (size of the bubbles, coalescence, etc.) was judged visually. The measurements were performed in duplicate.

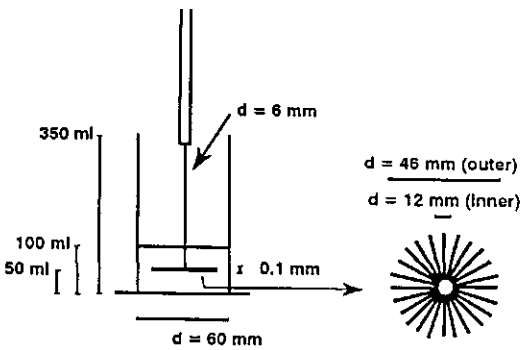

Figure 2. Schematic representation of the experimental setup for the foam screening test with a detail of the small impeller used. d = diameter.

Emulsions were made by mixing 2 mL tricaprylin oil (Sigma) and 18 mL 0.44% (w/v) protein solution for 1 min at 10000 rpm with a Polytron PT-MR-3000 homogeniser (Kinematica AG, Switzerland). This pre-emulsion was homogenised in a small-scale high-pressure homogeniser similar to the valve homogeniser described by Tornberg and Lundh (1978), by applying two passages at 60 bar. At several times (t = 0, 1 and 24 hour) after homogenising, emulsion samples were diluted (1:100 v/v) into an SDS-solution (0.1% w/v) to delay the instability processes (Pearce and Kinsella, 1978). The emulsion-forming ability was investigated by measuring the particle-size distribution (d_{32}) using the Malvern MasterSizer X (Malvern Instruments Limited, UK), with optical parameters defined by the manufacterer's standard presentation code, immediately after homogenising (t = 0 hour). The instability of the emulsions was estimated by measuring the decrease of the turbidity at 500 nm (Pearce and Kinsella,

1978) for all samples collected. Furthermore, the emulsion samples taken were examined for the presence of flocs and/or aggregates using a Polyvar light microscope (Reichert-Jung, Austria) at a magnification of 400.

The influence on the surface pressure at the A/W interface by the various protein solutions (20 mg/L) was examined by measuring the rate of lowering the surface tension using a Langmuir trough setup as described by Van Aken and Merks (1996).

Results

βCN hydrolysis and fractionation

Plasmin hydrolysis of βCN and fractionation of the hydrolysate resulted in eight peptide fractions as outlined in Figure 1. Figure 3 shows RP-HPLC patterns of these fractions. By using the RP-HPLC gradient described we were best able to separate the genetic variants of βCN and some of the hydrophobic βCN peptides formed after plasmin hydrolysis (Figure 3), although the gradient caused rather broad peaks at retention times between approximately 40 and 60 min. Although not shown in Figure 3, PEL-2 and PEL-3 were analysed by RP-HPLC as well: PEL-2 consisted mainly of group V peptides (see below), thereby resembling PEL-1; the ethanol precipitation was not very selective, and consequently resulted in a rather non-specific peptide composition of PEL-3 (results not shown). The hydrolysis and fractionation has been repeated several times, and the peptide composition (as analysed by RP-HPLC) in the several fractions obtained was very reproducible.

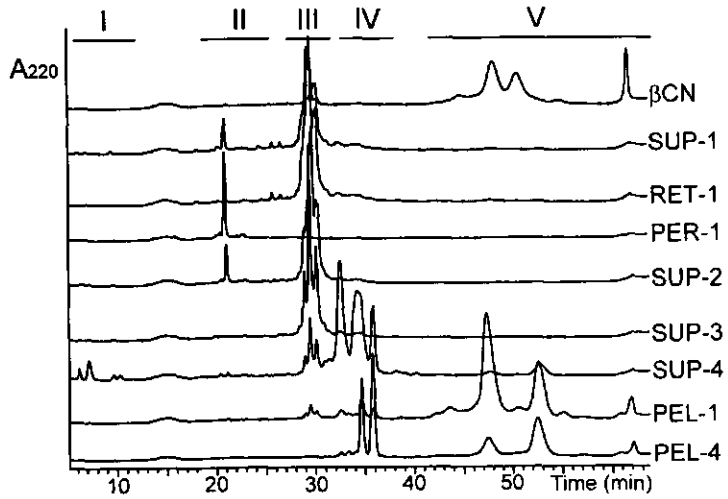

Figure 3. RP-HPLC chromatograms of the βCN hydrolysate fractions: for abbreviations see Figure 1. Peptide composition of the fractions categorised in groups I-V (Visser et al., 1989).

The peptides were categorised into five groups (I - V), indicated in Figure 3, according to Visser et al. (1989). Peptides in group II were identified as part of the hydrophilic N-terminus of βCN (sequence 1/2-25/28); peak material present in group III appeared to originate from the middle part of βCN (sequence 33/49-97/99); peak material present in group V was identified as peptide material from the C-terminal half of βCN (sequence 106/114-209); and peaks present in groups I and IV were hydrolysis products of peptides from group V (Visser et al.,1989). Table 1 shows the occurrence of these groups in the various βCN hydrolysate fractions mentioned in Figure 1, as well as the yield of the several βCN hydrolysate fractions.

Table 1. Yield of the βCN hydrolysate fractions (Figure 1) and peptide composition of these fractions categorised in groups I-V (Visser et al., 1989) in the RP-HPLC chromatogram.

Fraction[a]	Yield (% w/w)[b]	Group I	Group II	Group III	Group IV	Group V
SUP-1	53	–[c]	+[c]	+	(±)[c]	(±)
ultrafiltration:						
RET-1	46	–	–	+	–	(±)
PER-1	1.5	–	+	–	–	–
precipitation:						
PEL-2	6.8	–	–	±	(±)	+
SUP-2	49	–	+	+	–	–
PEL-3	23	–	+	+	(±)	–
SUP-3	20	–	–	+	–	–
PEL-1	38	–	–	±	±	+
hydrolysis:						
SUP-4	32	+	–	±	+	±
PEL-4	6.5	–	–	–	+	+

[a] for abbreviations see Figure 1

[b] based on the βCN starting material

[c] + present; – absent; ± traces

The hydrolysate fractions SUP-1, SUP-2, and RET-1 on the one hand, and SUP-3 on the other hand, seemed to vary in the composition of peptides belonging to group III (see Figure 3). Therefore, RET-1 and SUP-3 were also analysed with CE as shown in Figure 4. It appeared that RET-1 contained seven main peaks whereas SUP-3 contained four main peaks.

The MS results indicated that SUP-1 and SUP-3 contained a mixture of peptides (group III), of which four peptides could be identified: βCN[f 29-105] of genetic variants A^1 and A^2 (mass: 8757.2 and 8717.3 Da, respectively) and βCN[f 29-107] of the genetic variants A^1 and A^2 (mass: 9023.0 and 8981.7 Da, respectively). The MS results of PEL-1 indicated that the first, earlier eluting, main peak in group V (RP-HPLC) was βCN[f 106-209] (mass: 11823.2 Da) and the second main peak of group V was a mixture of βCN[f 108-209] and βCN[f 114-209] (mass: 11559.0 and 10829.0 Da,

respectively). The MS results of PER-1 showed that the only peak present (in group II; RP-HPLC) was βCN[f 1-28] (mass: 3478.0 Da, including 4 phosphoserines). These results are in agreement with the literature (Visser et al., 1989).

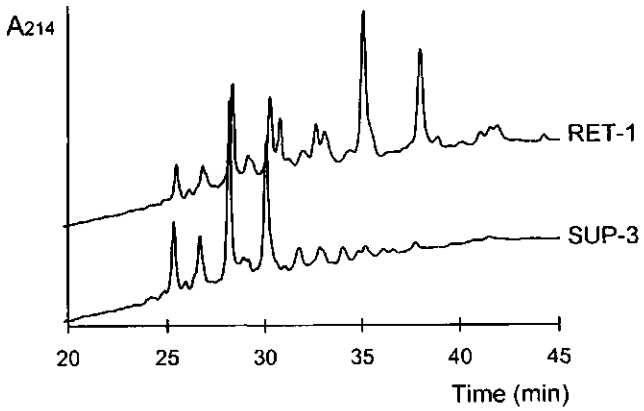

Figure 4. *CE electropherogram of the βCN hydrolysate fractions RET-1 and SUP-3: for abbreviations see Figure 1.*

Functional properties

Most of the fractions dissolved well in the buffers used. Only the hydrophobic fractions (PEL-1, SUP-4, and PEL-4) were poorly soluble at both pH values (as was βCN at pH 4.0). The reproducibility of the screening tests was investigated with the intact protein. The maximal difference in foam height was obtained for the initial amount of foam produced with the whipping method (maximal standard deviation ~ 5 a.u.). The standard deviation of the particle-size distribution (d_{32}) in the emulsion test was approximately 0.1 μm. The surface pressure curves, obtained with the Langmuir trough set-up were almost identical for the duplicate measurements. The screening tests used were reproducible enough to detect differences in functionality between the peptide fractions and the intact protein.

Figure 5 shows the results of the foam screening test. Differences were found in foam-forming ability: PEL-1 formed the highest amount of foam at pH 6.7, whereas at pH 4.0 SUP-3 and SUP-4 formed the highest amount of foam. At pH 4.0 βCN did not form a foam at all. The various peptide fractions produced foams with a clear variability in foam stability: PEL-1 (and βCN) formed the most stable foam at pH 6.7, while SUP-4 and PEL-4 were found to result in the highest foam stability at pH 4.0. The foams formed with PER-1 at pH 6.7, and with PER-1 and SUP-3 at pH 4.0, showed very rapid coalescence (PER-1 at pH 6.7 within 2 min). Furthermore, some visual characteristics of the several foams showed clear differences. The hydrophobic fractions (PEL-1, SUP-4, and PEL-4) formed "flocculated" foams at pH 6.7. At this pH,

PEL-1 and βCN showed no coalescence, while at pH 4.0 PEL-1, SUP-4, and PEL-4 showed no coalescence. After approximately 20 min the foams of these hydrophobic fractions had a rather dry appearance.

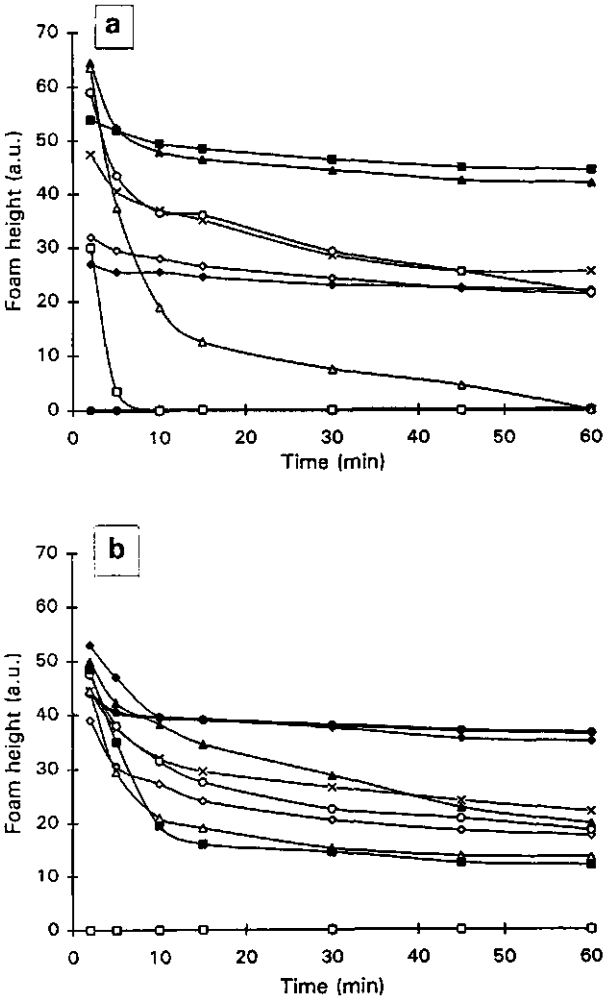

Figure 5. Foam height as produced with the various βCN hydrolysate fractions, as a function of time after whipping (means of duplicate measurements). pH 4.0 (a) and pH 6.7 (b). (●) βCN; (◇) SUP-1; (◆) PEL-1; (×) RET-1; (□) PER-1; (○) SUP-2; (△) SUP-3; (▲) SUP-4; (■) PEL-4; for abbreviations see Figure 1.

Table 2 shows the results of the emulsion screening test at pH 6.7. The emulsifying properties of the eight fractions tested were diverse: the relatively hydrophilic fractions (SUP-1, RET-1, PER-1, SUP-2, and SUP-3) formed smaller

emulsion particles than intact βCN, whereas the more hydrophobic fractions (PEL-1, SUP-4, and PEL-4) formed flocculated emulsions. Because of this flocculation, the measurement of the particle-size distribution was rather inaccurate, and was, therefore, not taken into account. By microscopic observation, however, it was shown that these hydrophobic, poorly soluble peptides were able to form rather small emulsion droplets (diameter approximately 2-3 μm), most of which were flocculated (Figure 6). The stability of the emulsions formed with SUP-1, SUP-2, and RET-1, which all mainly contained peptides from group III (Figure 3), was slightly lower than that of the βCN emulsions. Fraction SUP-3, containing only peptides from group III, and PER-1, containing only peptides from group II, formed emulsions that completely separated by coalescence within 1 hour. At pH 4.0 all fractions, except SUP-4 and PER-1, formed flocculated emulsions, and the particle-size distributions could therefore not be measured with the Malvern MasterSizer X. The emulsion made with PER-1 formed emulsion droplets of about 3 μm, and this emulsion creamed within 1 hour. Fraction SUP-4 formed emulsion droplets of approximately 1.8 μm. The emulsion of SUP-4 flocculated when dispersed in the SDS-solution; consequently the turbidity results could not be meaningfully interpreted. However, this emulsion was rather stable (no visible oil separation, and no increased particle size after 2 days, measured with the Malvern MasterSizer X).

Table 2. Screening-test results of emulsions made with βCN hydrolysate fractions[a].

Fraction[b]	d_{32} (μm)[c]	Stability
βCN	2.2	+++[d]
SUP-1	1.5	+
PEL-1	n.d.[e]	flocculation
RET-1	1.4	++
PER-1	1.5	coalescence
SUP-2	1.9	+
SUP-3	1.7	coalescence
SUP-4	n.d.	flocculation
PEL-4	n.d.	flocculation

[a] for conditions used see Materials and Methods section

[b] for abbreviations see Figure 1

[c] d_{32} is the particle-size distribution of the emulsion droplets

[d] +, ++, +++ indicate the extent of stability

[e] n.d. means not determined

Figure 7 shows the increase of surface pressure of the buffered peptide solutions. At pH 6.7 all peptide fractions were soluble at the low concentrations used. The rate by which the surface pressure increased varied among fractions: SUP-4 increased the surface pressure very rapidly (even faster than βCN), while PEL-1 and PEL-4 were

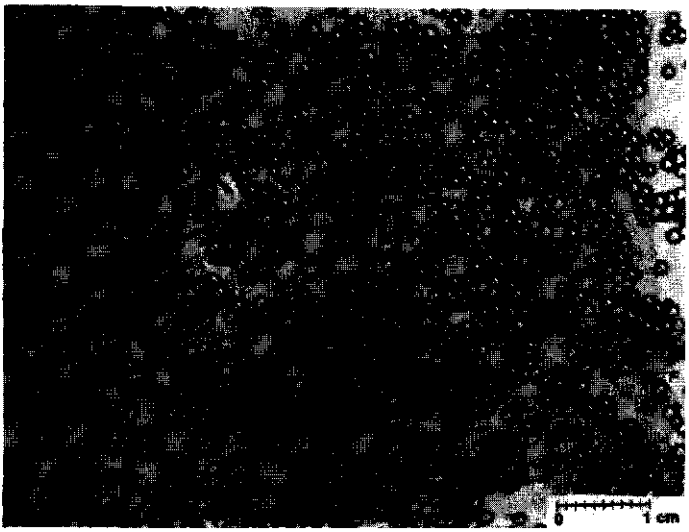

Figure 6. Microscopic observation of flocculated emulsion droplets of emulsions made with PEL-1; magnification 400×.

slower than βCN. The surface pressures of the hydrophobic fractions (PEL-1, SUP-4, and PEL-4) and intact βCN were almost identical after approximately 1 hour, the surface pressure of PER-1 was low, and the surface pressures of SUP-1, SUP-2, SUP-3, and RET-1 reached approximately half the value of βCN. At pH 4.0 both βCN and PEL-1 were not completely soluble (even at the low concentrations used), and the adsorption curves of these fractions only give an indication of their surface-active behaviour under these conditions. It appeared that the initial surface pressure of the βCN-solution increased very rapidly (possibly due to the presence of traces of surface-active material other than βCN), after which the increase of the surface pressure delayed. At this acidic pH the hydrophobic fractions (PEL-1, SUP-4, and PEL-4) reached the highest surface pressure. βCN and PER-1 reached almost the same final surface pressure as SUP-1, SUP-2, SUP-3, and RET-1, and this value was similar to the final surface pressure of these fractions at pH 6.7.

Discussion

Under the conditions used for hydrolysis and fractionation it is possible to produce several fractions from βCN that distinctly differ in peptide composition. With these mild hydrolysis conditions βCN could be degraded into mainly three parts: more hydrophobic fractions (PEL-1, SUP-4, and PEL-4), amphipathic fractions (SUP-1, SUP-2, SUP-3, and RET-1), and a strongly hydrophilic fraction (PER-1).

βCN is an amphipathic protein with a fairly distinct segregation of charged and hydrophobic residues (Swaisgood, 1982). The C-terminal half of the sequence,

corresponding to some γCNs, is very hydrophobic, which will be the reason for its precipitation at higher temperatures. This feature was utilised for the hydrolysis with intermediate pellet separation. The hydrophobicity of these γCNs may also result in a high surface pressure induced by these peptides, which has already been reported in the literature (Wilson et al., 1989). Our results also demonstrate an important influence on the surface pressure of the hydrophobic fractions (PEL-1, SUP-4, and PEL-4; Figure 7), at both pH 6.7 and pH 4.0. At pH 6.7 these fractions formed flocculated foams and emulsions, which may be caused by strong interactions between the hydrophobic peptides present. However, when made at 4°C (at which temperature the

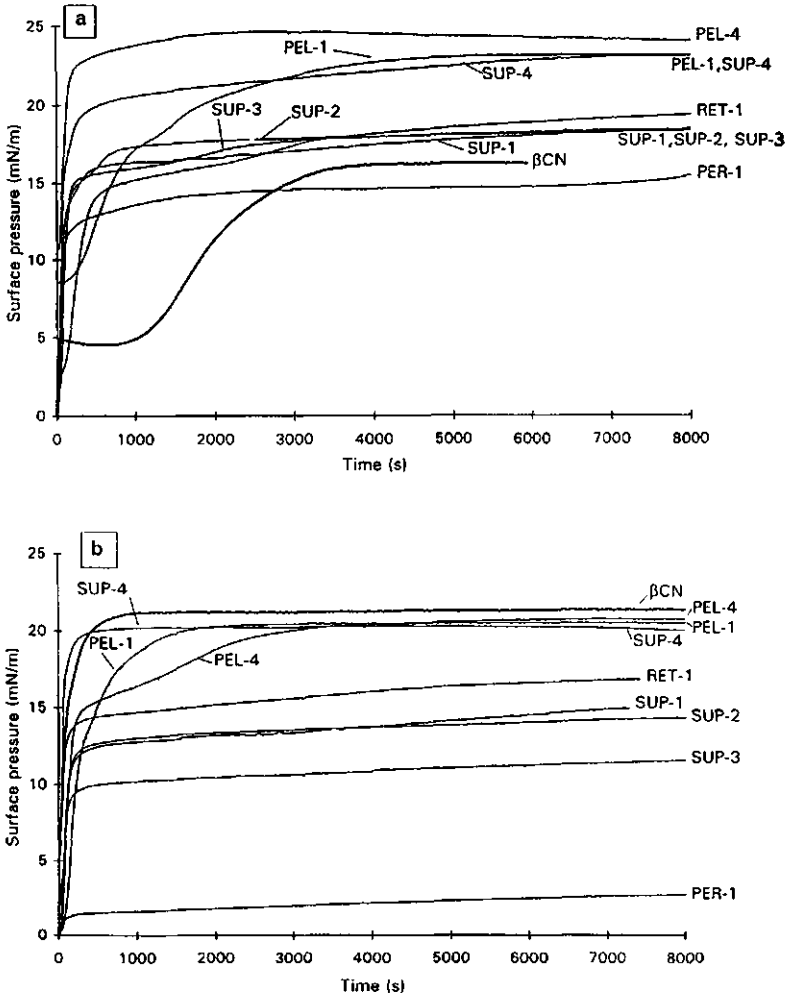

Figure 7. Surface pressure induced by βCN hydrolysate fractions as a function of time at an air/water interface measured using a Langmuir trough: for abbreviations see Figure 1. pH 4.0 (a) and 6.7 (b).

solubility of SUP-4 was increased, and PEL-1 and PEL-4 were still poorly soluble), the emulsions of the hydrophobic fractions were also flocculated. Apparently, the hydrophobic interactions are not the only driving force for flocculation. Another possible explanation for flocculation is that solid particles of the hydrophobic fractions, which were observed at pH 6.7, cause bridging flocculation of the droplets or bubbles (Walstra, 1987). A further possibility is that the peptides, present in these fractions, cannot generate enough repulsion between the emulsion droplets and/or foam bubbles. This would result in thin films between the droplets/bubbles, which may cause flocculated systems.

At pH 4.0 all peptide fractions had enhanced foaming properties as compared to intact βCN. Especially the hydrophobic fractions (PEL-1, SUP-4, and PEL-4) produced stable foams at pH 4.0 (Figure 5), which did not flocculate; this suggests an interesting possibility to apply these kinds of peptides in fluffy, acid foods.

The hydrophilic fraction (PER-1) showed almost no influence on the surface pressure at pH 6.7. At pH 4.0 the surface pressure induced by PER-1 increased dramatically compared to the surface pressure at pH 6.7; similar effects for βCN[f 1-25] have been reported in the literature (Lee et al., 1987a). At this acidic pH, the peptide will be less charged, because it is closer to its iso-electric point (pI; calculated pI value βCN[f 1-25] = 1.68; Nau et al., 1995). The inferior foam and emulsion-stabilising properties of PER-1, especially at pH 6.7, could be related to the low surface pressure induced at this pH. Furthermore, it is generally accepted that peptides must have a certain minimal molecular weight (MW) to retain some functionality (Turgeon et al., 1992), and the small size of the peptide could, therefore, be an additional reason for its poor functional properties at both pH's tested.

The amphipathic fractions (SUP-1, SUP-2, SUP-3, and RET-1), all containing peptides from the central part of the βCN sequence (group III), were expected to possess interesting foam, emulsion and surface-active properties; this expectation arises from their amphipathic character, and because of their rather high MW (about 9000 Da). It appeared that these fractions increased the surface pressure, but not to the extent as observed with βCN at pH 6.7. The fractions were able to form and stabilise a foam, although coalescence occurred: especially the foam made with SUP-3 at pH 4.0 showed rapid coalescence. At pH 6.7 the amphipathic fractions possessed improved emulsion-forming ability, as they formed smaller emulsion droplets than βCN did. The emulsion stability of these fractions varied significantly. As SUP-3 (unstable emulsion) is a more purified fraction than SUP-1, SUP-2, and RET-1 (stable emulsion), these results may suggest a synergistic effect between peptides present in the latter fractions. This has also been reported in the literature (Shimizu et al., 1986; Lee et al., 1987b). However, this functionality can also be produced by one or more particular peptides of group III (RP-HPLC) which were present in RET-1 (and probably also in SUP-1 and SUP-2) but absent in SUP-3 (Figure 4).

At pH 4.0 βCN has poor emulsifying properties. All peptide fractions, except for SUP-4 and PER-1, formed flocculated emulsions at acidic pH. PER-1 led to rather large emulsion droplets, which creamed rapidly; only SUP-4 formed a stable emulsion

at this pH. From these results it can therefore be concluded that no general rule exists for good emulsion properties of peptides at acidic pH with respect to hydrophobicity only.

In the present study the influence of plasmin hydrolysis of βCN on the foam, emulsion and surface-active properties was tested. The methods used to investigate these functional properties are quite suitable to screen for differences in functionality between the various peptide fractions. However, at this moment no information is available yet on the mechanisms responsible for these functional properties.

In conclusion, our results show that by plasmin hydrolysis of βCN and fractionation of the hydrolysate well-defined peptide mixtures can be produced which clearly vary in emulsion, foam and surface-active properties, as judged by the results of the screening tests. Especially, large variability was found for foam stability at pH 4.0, and for emulsion stability at pH 6.7.

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Chapter 3

Functionality of β-casein peptides: importance of amphipathicity for emulsion-stabilising properties

Abstract

Peptides from bovine β-casein (βCN), derived by plasmin hydrolysis and fractionation of the hydrolysate, were isolated and identified based on their masses determined by electrospray-ionization mass spectrometry, the primary structure of the intact protein, and the known specificity of the enzyme. An amphipathic peptide fraction was fractionated further by ion-exchange chromatography and subsequent hydrophobic-interaction chromatography resulting in the components βCN[f 1-105/107] and βCN[f 29-105/107]. The latter peptides had poor emulsion-stabilising properties compared to the former ones, and the stability of an emulsion formed with βCN[f 29-105/107] was also more sensitive to hydrophobic impurities than that of an emulsion formed with βCN[f 1-105/107]. The highly-charged N-terminal part appeared to be important for the emulsion-stabilising properties of these peptides. An hypothesis for the structure-function relationship is given.

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Introduction

Milk proteins are known for their ability to form and stabilise foam and emulsions (Walstra, 1988; Wong et al., 1996). Enzymatic hydrolysis can be used to modify such functional properties (Arai and Fujimaki, 1991; Chobert et al., 1996; Panyam and Kilara, 1996). Shimizu et al. (1984; 1986) investigated the effect of hydrolysis on the emulsifying properties of α_{s1}-casein. They demonstrated that the emulsifying activity of a non-pure fraction containing mainly the α_{s1}-casein fragment 1-23 decreased after subsequent purification of the sample. It was, therefore, concluded that this functionality was in fact formed by a so-called synergistic effect with coexisting peptides present in the sample (Shimizu et al., 1986). Lee et al. (1987) reported similar synergistic effects between the β-casein fragment 193-209 and the κ-casein fragment 106-169 (glycomacropeptide). By the method used in these papers (emulsion activity index according to Pearce and Kinsella, 1978) no information could be obtained about the stability of the emulsions formed.

One of the major caseins present in milk is β-casein (βCN), a 24 kDa protein with a distinct distribution of charged groups, and little secondary structure. The N-terminal part of the sequence is highly hydrophilic and contains 5 phosphoseryl residues (residues 15, 17, 18, 19 and 35), while the C-terminal part contains mainly hydrophobic side chains, carrying almost no net charge at neutral pH (Swaisgood, 1982).

In Chapter 2 we described the foam- and emulsion-forming and -stabilising properties as well as the surface-active properties of βCN peptide mixtures, which were produced by plasmin hydrolysis of βCN. By applying a stepwise fractionation of the hydrolysate we were able to test the influence of removing certain peptides on the functional properties of the fractions. The final fractions obtained could be categorised as hydrophilic, amphipathic and hydrophobic fractions showing differences in functional properties. Moreover, differences were observed between the emulsion-stabilising properties of the various amphipathic fractions. The results might be explained by the above described so-called synergistic effects between the different peptides present in the fractions. However, this functionality may also be accomplished by one or more specific peptides present in some peptide fractions, but absent in others (Chapter 2).

In the study presented in this Chapter the peptides in the previously described βCN peptide fractions were identified in order to establish structure-function relationships. To investigate the possibility of either synergistic effects or specific functional properties of certain peptides, an amphipathic fraction was fractionated further, and the peptides obtained were analysed for their composition and emulsion-forming and -stabilising properties.

Materials and Methods

Materials

Bovine βCN (90% w/w βCN, 95% βCN based on nitrogen, w/w), containing mainly the genetic variants A^1 and A^2, was purchased from Eurial (France). Bovine plasmin (EC 3.4.21.7) and aprotinin were obtained from Sigma (USA; article numbers P-7911 and A-6012, respectively). Unless stated otherwise, all other chemicals were of analytical grade, and were purchased from Merck (Germany) or BDH (UK).

Preparation of β-casein peptides

The preparation of the βCN peptide fractions has been described earlier (Chapter 2). Briefly, the preparation was as follows. Bovine βCN was hydrolysed at pH 6.8 by bovine plasmin with intermediate pellet separation. The total pellet fraction (PEL-1) was dissolved at pH 10, after which part of it was hydrolysed further at pH 8 with plasmin. Hydrolysis was terminated by adding aprotinin to the reaction mixture. The two resulting hydrolysates (the supernatant of the βCN-hydrolysate, SUP-1, and the hydrolysate of PEL-1) were fractionated by means of selective precipitation based on temperature, pH and solvent composition (SUP-2, SUP-3, PEL-4 and SUP-4) and by ultrafiltration (RET-1 and PER-1).

Ion-Exchange Chromatography (IEC)

Preparative IEC was performed on an ÄKTA-explorer, controlled by a UNICORN-control system (Pharmacia Biotech, Sweden) using a SourceQ column (280 mL bed volume; Pharmacia) at 20°C. Solvent A (20 mM Tris/HCl buffer, pH 8) and solvent B (20 mM Tris/HCl buffer, 1 M NaCl, pH 8) formed the eluent in the following linear gradient steps: 5 min isocratic elution at 100% A; 2 min sample-injection (25 mL/min); to 50% B over 10 min; to 100% B over 3 min followed by 3 min isocratic elution at 100% B; finally to 100% A over 3 min. Except where stated otherwise, a flow rate of 60 mL/min was applied. Appropriate IEC fractions were pooled, desalted by ultrafiltration (OMEGA membrane 5 kDa MWCO, Pall Filtron Corp., USA), lyophilised, and stored at 4°C prior to analysis or further purification. Analytical IEC runs were performed on an FPLC system (Pharmacia) using a ResourceQ column (1 mL, Pharmacia) at 20°C. Solvents and gradient for the analytical runs were similar to those for the preparative runs, except for the flow rate, which was 1 mL/min, and the injection volume, which was 200 μL. Generally, the protein load of the column was 1 mg per mL bed volume for both preparative and analytical runs, and detection was at 220 and/or 280 nm.

Hydrophobic-Interaction Chromatography (HIC)

HIC was performed on a High-Load system (Pharmacia) using a Phenyl Sepharose High Performance column (150 mL bed volume, Pharmacia) at 4°C. Solvent A (50 mM potassium phosphate buffer containing 1.7 M ammonium

sulphate, pH 7.0) and solvent B (50 mM potassium phosphate, pH 7.0) formed the eluent in the following linear gradient steps: 2 min isocratic elution at 100% A; to 48% B over 19.4 min; to 85% B over 26.5 min; to 100% B over 2.1 min and finishing by 4 min isocratic elution at 100% B. A flow rate of 28 mL/min was applied, and detection was performed at 280 nm. The samples to be purified by HIC were collected from the preparative IEC runs, and diluted 3-fold in the sample solvent (75 mM potassium phosphate buffer containing 1 M ammonium sulphate, pH 7.0). The protein load of the column was approximately 1 mg per mL bed volume. Prior to loading, the column was equilibrated with solvent A for 5 min at 28 mL min^{-1}. Appropriate HIC fractions were pooled, desalted (ultrafiltration 5 kDa MWCO), lyophilised, and stored at 4°C prior to analysis.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The peptide composition of the βCN peptide fractions was analysed by RP-HPLC. The equipment used was described by Visser et al. (1991). For the separation of the several peptide fractions two different gradients, formed with solvent A (0.1% trifluoroacetic acid [TFA] in 10% aqueous acetonitrile, v/v) and solvent B (0.08% TFA in 90% aqueous acetonitrile, v/v), were used. Gradient RP-1 was used for the total βCN peptide fractions, and has been described previously (Chapter 2). Gradient RP-2 was used for the analysis of amphipathic βCN peptide fractions, and the linear gradient steps to form gradient RP-2 were as follows: from 25% to 30% B over 3 min followed by 6 min isocratic elution; to 36% B over 12 min; then to 70% B over 5 min and finally 5 min isocratic elution at 70% B. For both gradients, a flow rate of 0.8 mL/min was applied. Generally, 50 μL 1 mg/mL protein solution was injected onto the column for the analytical runs, and 100 μL 2 mg/mL for the semi-preparative runs. Peak detection and quantitation was performed at 220 nm using Turbochrom data-acquisition and processing software (Perkin-Elmer, Germany).

Electrospray-Ionization Mass Spectrometry (ESI-MS)

All βCN peptides were collected by semi-preparative RP-HPLC and identified by ESI-MS on a Quattro II triple-quadrupole instrument (Micromass, UK). Samples were dissolved in water, and this solution was diluted in a mixture of acetonitrile - water - formic acid (100 : 100 : 1, v/v/v) and injected into a flow of acetonitrile - water (1 : 1, v/v, 4 μL/min). The potential at the capillary tip was maintained at 3.2 kV; the cone voltage was 30 V. Nitrogen was used as nebulizing and drying gas. Calibration was performed using horse-heart myoglobin. The raw mass spectral data were processed and transformed with Masslynx software version 2.2 (Micromass). Peptides were identified from the molecular masses determined combined with sequence data of the protein and the known specificity of plasmin (Arg-X and Lys-X; Bastian and Brown, 1996).

Lysinoalanine (LAL) determination

Determination of the LAL content of the samples was performed as described elsewhere (Pellegrino et al., 1996).

Emulsion properties

Emulsion-forming and -stabilising properties of the peptides were tested in a screening test at pH 6.7, calculated ionic strength 0.075 mM and 20°C, as described earlier (Chapter 2). Briefly, the screening test was as follows. The emulsions (tricaprylin oil/water 1/9 v/v; 0.44% w/v protein solution) were made by homogenising in a laboratory high-pressure homogeniser at 60 bar. The emulsion-forming property was determined by measuring the droplet-size distribution immediately after homogenising using the Malvern MasterSizer X (Malvern Instruments Limited, U.K.). The emulsion-stabilising property against mainly coalescence was determined by measuring the turbidity at 500 nm immediately after homogenising, and again after 1 h and 24 h. The presence of flocs and/or aggregates in the emulsion samples taken was examined using a light microscope.

Results

Identification of peptides

Table 1 shows the ESI-MS results of the plasmin-derived βCN peptides. The code of the peaks refers to Figure 1, which shows the RP-HPLC chromatograms of the βCN fractions described previously (Chapter 2). The results (Table 1) show that the differences between the measured and calculated mass values are mostly within 2 Da. Therefore, the identification can be based on the masses determined, the primary structure of βCN, and the specificity of the enzyme. Nearly all peptides present in the fractions (Figure 1) could be identified, and the complete βCN sequence could be recombined from the peptide sequences present in the several fractions. The mass spectrum of peak V-x was difficult to interpret; the results displayed a complex mixture of different masses. This peak was also present in the starting material (Chapter 2). Further analysis showed that the material present in this peak contained approximately 900 ppm LAL (unpublished results), and hence it contained some cross-linked protein material, probably formed during the purification of the protein. On the basis of the molecular mass found it is likely that the fraction contained "βCN- and γCN-like material" (Table 1).

Purification of amphipathic peptide fractions

The several amphipathic peptide fractions SUP-1, RET-1, SUP-2 and SUP-3 (Figure 1) had distinct differences in emulsion-stabilising properties (Table 2). In our previous study (Chapter 2) it was shown that fraction SUP-3 (having poor emulsion-stabilising properties) contained fewer peptides than fraction RET-1 (having fairly good emulsion-stabilising properties). Therefore, RET-1 was further fractionated by

Table 1. ESI-MS results for the RP-HPLC collected peak components of βCN/plasmin hydrolysates (Figure 1).

RP-HPLC groups[a]	peak	Measured value (Da)	Peptide sequence	Calculated value (Da)
I	a	283.7	106-107	283.3
		779.3	170-176	780.0
	b	1012.5	106-113	1013.2
	c	747.4	108-113	747.9
		1949.4	-	
II	a	3478.0	1-28	3478.4
III	x	8716.0	A^2(29-105)	8716.9
		8755.7	A^1(29-105)	8756.9
		8980.9	A^2(29-107)	8982.2
		9021.3	A^1(29-107)	9022.3
		12175.1	A^2(1-105)	12177.3
		12216.0	A^1(1-105)	12217.3
		12441.0	A^2(1-107)	12442.6
		12480.0	A^1(1-107)	12482.6
IV	a	4483.4	170-209	4484.4
	b	3721.3	177-209	3722.5
	c	7356.4	106-169	7357.6
		8118.3	106-176	8119.5
	d	4483.0	170-209	4484.4
		6360.7	114-169	6362.3
		7122.5	114-176	7124.3
		7852.5	108-176	7854.2
	e	3721.3	177-209	3722.5
		7090.0	108-169	7092.2
V	a	11823.0	106-209	11824.0
	b	10827.1	114-209	10828.7
		11556.7	108-209	11558.6
	x	24044.0	-	
		24056.9	-	
		23590.0	-	
		11555.0	108-209	11558.6
		6603.6	-	

[a] see Visser et al. (1989) for categorisation of RP-HPLC groups I-V

- no sequence could be identified

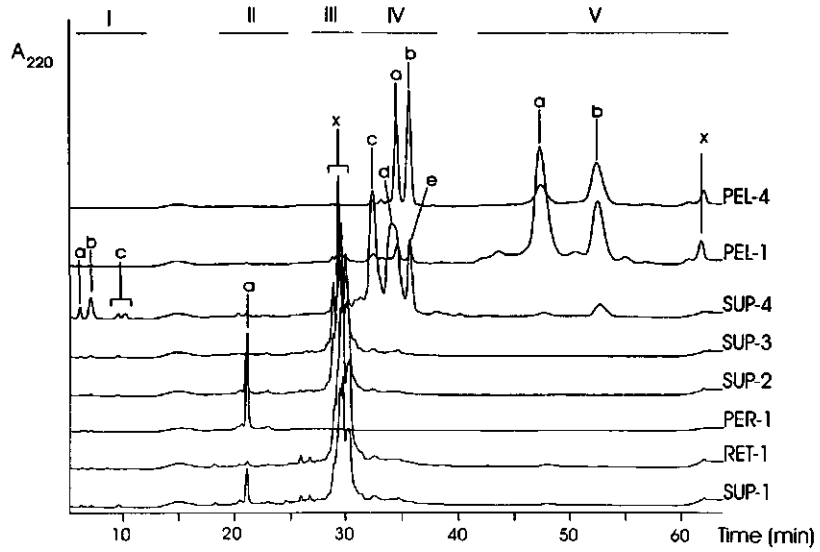

Figure 1. RP-HPLC chromatograms of the βCN hydrolysate fractions (Chapter 2); for abbreviations see text; RP-HPLC gradient RP-1. Peptide composition of the fractions is categorised in groups I-V (Visser et al., 1989). Code of the peaks refers to Table 1.

IEC, resulting in IEC1, IEC2 and IEC3 (Figure 2a). Analytical IEC separation of SUP-3 (results not shown) demonstrated that this fraction lacks the IEC3-peak (identified as βCN[f 1-105/107]). Figure 2b shows the RP-HPLC results of the IEC fractions in combination with the ESI-MS identified sequences of the amphipathic peptides. From the results it appears that IEC1 was a mixture of peptides from the C-terminal, hydrophobic part of βCN (RP-HPLC groups IV and V, Table 1). The main peptides present in IEC2 were βCN[f 29-105/107], whilst the main peptides in IEC3 were βCN[f 1-105/107].

The RP-HPLC chromatograms of IEC2 and IEC3 (Figure 2b) still showed peaks with a retention time of approximately 35 min (approximately 5% of the total RP-HPLC peak area determined at 220 nm), which is similar to the retention time of the hydrophobic peptides in the IEC1 fraction. To remove these last impurities from the IEC2 and IEC3 fractions, parts of these fractions were further purified by HIC (Figure 3a), resulting in a separation of the peptides into two fractions: IEC2HIC + HIC-R and IEC3HIC + HIC-R, respectively. Figure 3b shows the RP-HPLC chromatograms of IEC-HIC-purified fraction. From the chromatograms it appears that after HIC purification most of the material eluting in the RP-HPLC chromatogram at 35 min was removed from IEC2 and IEC3 with the HIC-R fractions. The HIC-R fraction accounted for approximately 20% of the total HIC peak area determined at 280 nm, being about 4 times higher than analysed at 220 nm (Figure 2b). This could indicate

Table 2. A summary of the emulsion properties of βCN hydrolysate fractions described in Chapter 2.

Sample	Process	Emulsion[a,b,c] formed at pH 4		Emulsion[a,b,c] formed at pH 6.7	
β-casein	formation	n.d.	floc	2.2	
	stabilisation			+++	
SUP-1	formation	n.d.	floc	1.5	
	stabilisation			+	
RET-1	formation	n.d.		1.4	
	stabilisation		floc	++	
PER-1	formation	3.0		1.5	
	stabilisation	-	cr/floc	--	coal
SUP-2	formation	n.d.	floc	1.9	
	stabilisation			+	
SUP-3	formation	n.d.	floc	1.7	
	stabilisation			--	coal
PEL-1	formation	n.d.	floc	n.d.	floc
	stabilisation				
SUP-4	formation	1.7		n.d.	floc
	stabilisation	+			
PEL-4	formation	n.d.	floc	n.d.	floc
	stabilisation				

[a] - and + indicate the extent of emulsion stabilisation, based on turbidity measurements (from stable [+++] to complete phase separation within 8 hours [--])

[b] coal: coalescence; floc: flocculation; cr: creaming

[c] average droplet size (μm) immediately after homogenising

n.d. not determined because of flocculation

a relatively high content of aromatic residues in these fractions. The protein material with a retention time of approximately 35 min was collected from IEC2, IEC3 and HIC-R (see Figure 2b and 3b) by semi-preparative RP-HPLC and analysed by ESI-MS. The mass spectra were difficult to interpret. Mass ranges were from 16055 Da to 16855 Da in IEC2, and from 19515 Da to 24070 Da in IEC3. Masses deduced from the ESI-MS spectrum of HIC-R were 5366 Da, and from 16058 Da to 16860 Da. The results obtained with these fractions did not allow a determination of βCN sequences. Before IEC purification, these masses were not found among peptides of similar retention times (IEC1), probably because the amounts present were too low to detect them among the other peptides present. Analytical re-injection of the removed HIC-R on the IEC-column resulted in two peaks with retention times similar to those of IEC2 and IEC3 (see Figure 2), indicating that the protein material present in HIC-R resembles the IEC-fractions mentioned (results not shown). The material in this fraction appeared to contain 1300 ppm LAL. Apparently, the LAL content present in the starting material was somewhat concentrated in IEC2 and IEC3, and, after further purification, in HIC-R.

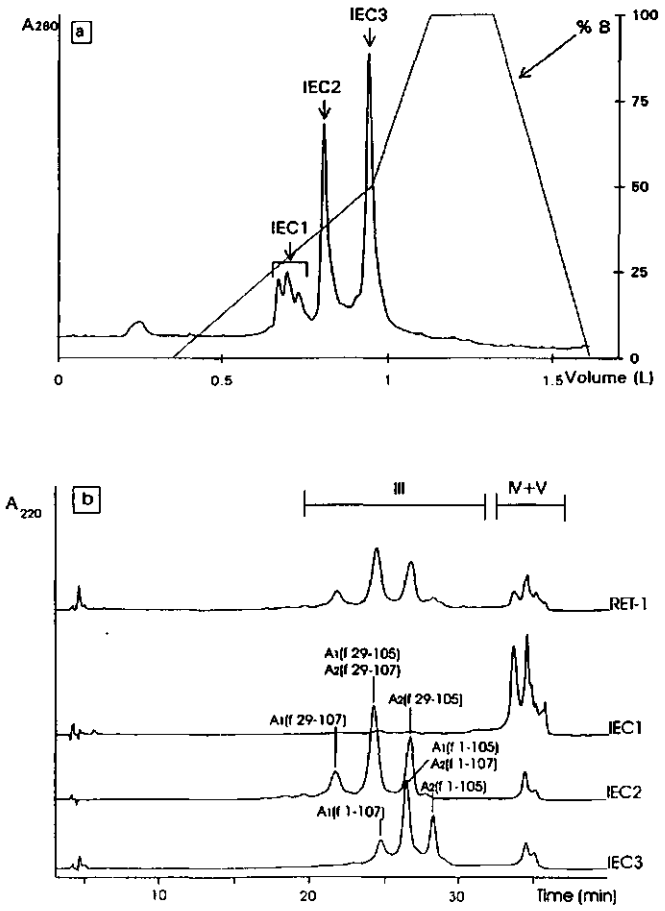

Figure 2. IEC chromatogram of RET-1 (a) and RP-HPLC chromatograms of the IEC-purified fractions (b): for abbreviations see text; RP-HPLC gradient RP-2. Peptide composition of the fractions is categorised in groups III-IV/V (Visser et al., 1989).

Emulsion-stabilising properties

Table 3 shows the results of the emulsion screening test with intact βCN, the crude fractions RET-1 and SUP-3, the IEC-fractionated IEC2 and IEC3 fractions, and the HIC-purified IEC2HIC and IEC3HIC fractions. The emulsion-forming properties of the peptide fractions were similar, and were superior to those of intact βCN, but the stabilising properties varied. Normally, smaller emulsion droplets cause slower coalescence (Walstra, 1996). However, the results in Table 3 show that, despite the smaller droplet size, the emulsions formed with the peptides are less stable against coalescence than the emulsion formed with intact βCN. RET-1 had fair stabilising properties, whereas SUP-3 showed poor stabilising properties, similar

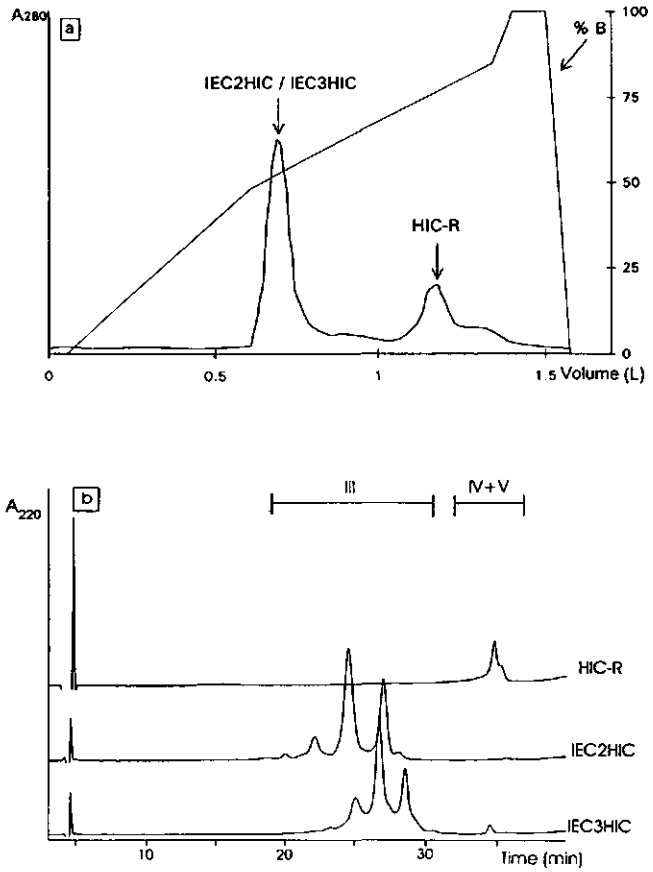

Figure 3. HIC chromatogram of IEC2 and IEC3 (a) and the RP-HPLC chromatograms of the HIC-purified fractions (b): for abbreviations see text; RP-HPLC gradient RP-2. Peptide composition of the fractions is categorised in groups III-IV/V (Visser et al., 1989).

to results obtained earlier (Chapter 2). IEC2 had poor stabilising properties as well, whereas IEC3 had fairly good stabilising properties, similar to those of RET-1 (both somewhat less than those of intact βCN). Since IEC2 and IEC3 are both fractions originating from RET-1, these results demonstrate the role of a specific peptide sequence rather than that of some synergistic effect between fragments in the emulsion stability. However, in this respect the results of IEC2HIC and IEC3HIC are less clear; the emulsion formed with IEC2HIC is only slightly less stable than that formed with IEC3HIC, which was comparable to that formed with IEC3. Hence, the removal of traces of impurities by HIC improved the stabilising properties of IEC2 (βCN[f 29-105/107]-enriched). Possibly, the material that was removed by HIC had a

negative influence on the emulsion-stabilising properties of the IEC fractions. Apparently (see Table 3), this destabilising effect was noticed in IEC2 (βCN[f 29-105/107]-enriched), but not in IEC3 (βCN[f 1-105/107]-enriched).

Table 3. Screening-test results of emulsions made with βCN hydrolysate fractions and IEC-purified fractions.

Fraction[a]	Sequence	d_{32}[b] (μm)	Stability[c,d]	
βCN	1-209	2.2	+++	
RET-1		1.5	++	
SUP-3		1.7	--	coal
IEC2	29-105/107	1.5	-	coal
IEC3	1-105/107	1.5	++	
IEC2HIC	29-105/107 purified	1.7	+	
IEC3HIC	1-105/107 purified	1.5	++	

[a] for abbreviations, see text

[b] average droplet size of the emulsion droplets immediately after homogenising

[c] - and + indicate the extent of emulsion stability against coalescence, based on turbidity measurements (from stable [+++] to complete phase separation within 8 hours [--])

[d] coal: coalescence

To investigate the influence of hydrophobic impurities on the emulsion-stabilising properties of the amphipathic peptides, βCN[f 1-105/107] and βCN[f 29-105/107], emulsions were made with IEC2, IEC3, IEC2HIC and IEC3HIC, containing 2.5-10% IEC1, which is a mixture of hydrophobic peptides. The results are shown in Table 4. Making emulsions with only 75% of the normal protein/peptide concentration of the amphipathic fraction SUP-1 (a mixture of mainly βCN[f 1-28] and βCN[f 1/29-105/107], and some traces of hydrophobic peptides) did not have a clear influence on the emulsion stability (no further results shown). Therefore, we concluded that the up to 10 % lower amount of amphipathic protein added in the experiments described will not have a major effect on the results. The stability of the emulsion formed with IEC2 (βCN[f 29-105/107]-enriched) was decreased even more after addition of IEC1. The addition of IEC1 to IEC3 (βCN[f 1-105/107]-enriched) had no negative influence on the emulsion stability against coalescence formed by this fraction. The fractions IEC2HIC and IEC3HIC were more sensitive to hydrophobic impurities. IEC2HIC with 2.5% w/w IEC1 addition was already unstable after 1 h, whereas IEC3HIC with 10% w/w IEC1 was unstable after 1 h. Despite this instability with 10% IEC1, IEC3HIC was rather stable during 1 h even with 7.5% w/w IEC1 addition. The emulsion-forming properties of the peptide mixtures decreased (i.e. average droplet size increased) when higher amounts of hydrophobic peptides were present. Possibly, the poor emulsion stability, caused by the hydrophobic peptides, influenced the determination of the average droplet size.

Table 4. Stability of emulsions made with mixtures of the several amphipathic peptide fractions and hydrophobic peptides.

Fraction[a,b]	d_{32}[c] (μm)	Turbidity after 1 h[d] (%)	Turbidity after 24 h[d,e] (%)
100%IEC2 + 0 % IEC1	1.5	61	20
90.0%IEC2 + 10.0%IEC1	2.3	31	11
100%IEC3 + 0 % IEC1	1.5	92	83
90.0%IEC3 + 10.0%IEC1	1.3	94	86
100%IEC2HIC + 0 % IEC1	1.7	71	45
97.5%IEC2HIC + 2.5%IEC1	1.7	3	-
95.0%IEC2HIC + 5.0%IEC1	2.7	2	-
92.5%IEC2HIC + 7.5%IEC1	4.1	2	-
90.0%IEC2HIC + 10.0%IEC1	4.1	2	-
100%IEC3HIC + 0 % IEC1	1.5	100	93
97.5%IEC3HIC + 2.5%IEC1	1.5	94	67
95.0%IEC3HIC + 5.0%IEC1	1.6	85	51
92.5%IEC3HIC + 7.5%IEC1	1.7	69	-
90.0%IEC3HIC + 10.0%IEC1	2.3	1	-

[a] for abbreviations, see text

[b] % in w/w

[c] average droplet size of the emulsion droplets immediately after homogenising

[d] % of turbidity remaining; turbidity at 0 h of emulsion formed with the fraction without IEC1 added set at 100%

[e] -: complete phase separation of the emulsion

Discussion

The emulsion results obtained (Table 3) with IEC2 (βCN[f 29-105/107]-enriched) and IEC3 (βCN[f 1-105/107]-enriched) suggest that the highly charged N-terminal end of the amphipathic peptide (1-28, containing 4 of the 5 phosphoserine residues present in βCN) is important for its stabilising properties. Also, the results (Table 2) with the crude fractions RET-1 (a mixture of mainly βCN[f 29-105/107] and βCN[f 1-105/107], and some traces of smaller hydrophobic peptides) and SUP-3 (similar to RET-I, but lacking βCN[f 1-105/107]) can be explained in this manner. The results indicate the importance of the hydrophilic N-terminus of the peptide βCN[f 1-105/107] for the emulsion-stabilising effects. Following these results, a hypothesis for the emulsion-stabilising effect of the highly charged N-terminus of the amphipathic peptides can be postulated. Apparently, the N-terminal part projects into the continuous phase, and the phosphoseryl groups present in this part of the molecule cause both electric and steric repulsion between the oil-droplets. As a result, this so called electro-steric repulsion of the N-terminal part prevents rapid coalescence, and produces stable emulsions. βCN [f 29-105/107] produces unstable emulsions, probably because it lacks the strong electro-steric repulsion of the

N-terminal part. HIC purification of βCN[f 29-105/107] improves the stabilising properties of this fraction (see Table 3). Before HIC treatment of the IEC fractions small traces of impurities are still present (approximately 5% of the total RP-HPLC peak area). It is assumed that, by analogy with the adsorption of the hydrophobic parts of intact proteins (Walstra and De Roos, 1993), these impurities adsorb to the interface with a higher preference than the amphipathic peptides, because of their somewhat higher hydrophobicity (the latter being judged by RP-HPLC retention time). Consequently, it can be hypothesised that less surface on the oil-droplet is available for the amphipathic peptides. Apparently, the peptides with the highly charged N-terminus can still cause a stable emulsion with a lower amount adsorbed, in contrast to the peptides with the shorter, less charged N-terminus. The results in Table 4 also indicate that the emulsions formed with amphipathic peptides with additional hydrophobic peptides present are less stable. Although the hydrophobic peptides also had some negative influence on the emulsion stability of emulsions formed with IEC3HIC (βCN[f 1-105/107]) the influence in the case of IEC2HIC (βCN[f 29-105/107]) was once again more dramatic.

Figure 4 depicts the hypothesis described above; Figure 4a illustrates the difference in conformation of βCN[f 1-105/107] and βCN[f 29-105/107] when adsorbed onto the interface, and Figure 4b demonstrates the effect of this difference on the emulsion-stabilising properties. The stronger amphipathic peptide βCN[f 1-105/107] causes more electro-steric repulsion between the emulsion droplets, and is, therefore, better capable of preventing coalescence of the emulsion droplets than the less amphipathic peptide βCN[f 29-105/107] is. This effect is evident for the IEC fractions. Both the HIC-purified IEC fractions, IEC2HIC (βCN[f 29-105/107]) and IEC3HIC (βCN[f 1-105/107]), formed fairly stable emulsions, similar to IEC3. The removal of the impurities allows a higher surface-load of the emulsion droplets with the amphipathic peptides, which results in sufficient repulsion between the emulsion droplets covered with βCN[f 29-105/107]. It has to be realised that the real distribution on the interface of the different peptides present in the IEC fraction is unknown, and the distribution shown in Figure 4b is tentative. However, it is clear that the hydrophobic impurities do disturb the emulsion-stabilising effect of the amphipathic peptides.

The hypothesis described above, concerning the electro-steric repulsion between the emulsion droplets caused by the amphipathic βCN peptides, concur with previous publications on the adsorption of intact βCN. It has, for instance, been reported (Leaver and Dalgleish, 1990; Ter Beek et al., 1996) that when βCN is adsorbed onto an oil/water interface, its highly hydrophilic N-terminus is flexible and protrudes into the water phase. Furthermore, neutron reflectivity studies revealed that the layer thickness of a βCN monolayer at n-hexane/water and/or at air/water interfaces is approximately 7-9 nm, which is larger than the layer thickness of 2-3 nm of some globular proteins such as β-lactoglobulin (βLg; Dickinson et al. 1993; Atkinson et al., 1995). Combining these results with the amount of protein adsorbed onto the interfaces [approximately 2.5-3.8 and 1.5-2 mg/m^2, for βCN and the globular

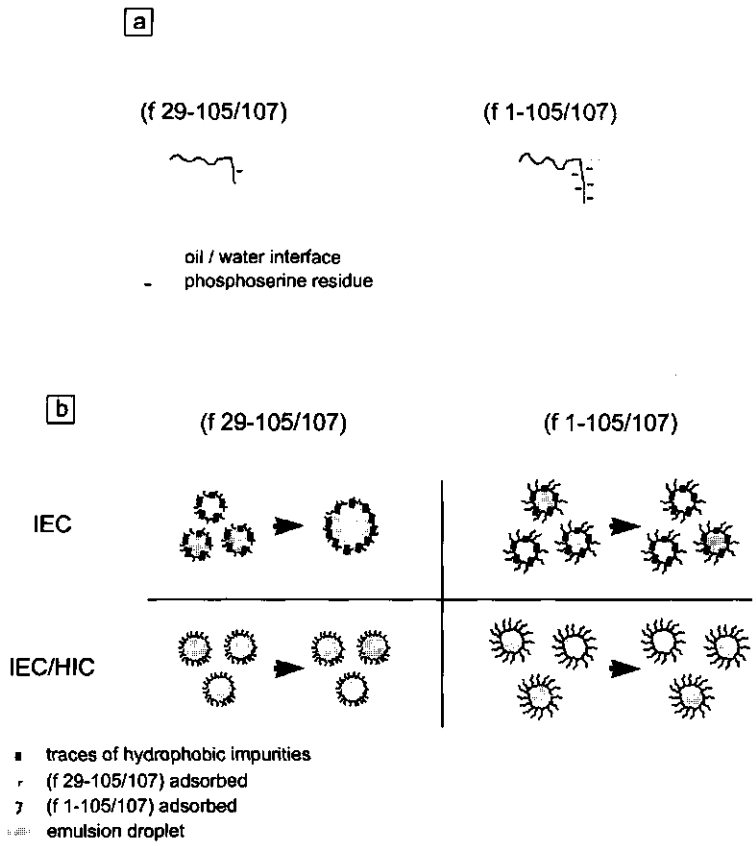

Figure 4. Schematic representation of the possible conformation of the amphipathic peptides at the oil/water interface (a) and schematic representation (not to scale!) of the hypothesis for the stabilising properties of the several amphipathic peptide fractions IEC2, IEC3, IEC2HIC, IEC3HIC (b); for abbreviations see text.

protein βLg, respectively (Dickinson et al. 1993; Atkinson et al., 1995; Smulders et al., 1998)], they can be explained by assuming that βCN adsorbs in a "loop and train" conformation, with a condensed layer directly at the interface (trains) and a more diffuse layer of the highly charged N-terminal part (loop and/or tail) further away from the interface (Dickinson et al., 1993; Nylander and Wahlgren, 1994).

Although the highly charged N-terminal part of the peptide appears to be critical for its emulsion-stabilising properties, the emulsion formed with the strongly amphipathic βCN peptide is less stable than that formed with intact βCN. Another study (Smulders et al., 1998) showed that the surface load of intact βCN on emulsion droplets is significantly higher than that of the amphipathic peptides (3.8 and 2.4 mg/m^2, respectively). The higher surface load found for intact βCN will

probably be caused by a condensed packing of the hydrophobic, C-terminal part of the molecule. Evidence for this is the high surface load of the peptide fraction PEL-1 (Table 2) on emulsion droplets (Smulders et al., 1998). The results in Table 1 show that the hydrophobic peptide fraction PEL-1 (Figure 1) contains peptides from the C-terminal half of βCN. Probably, this close packing of the C-terminal part of βCN is the reason for the better emulsion-stabilising properties of the intact protein compared to the amphipathic peptides, in spite of the somewhat larger droplet size.

In conclusion, our results show that the highly charged N-terminal part of the amphipathic βCN peptides is important for the stability against coalescence of emulsion droplets, especially when small traces of hydrophobic impurities are present.

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Chapter 4

The adsorption-induced secondary structure of β-casein and of distinct parts of its sequence in relation with foam and emulsion properties

Abstract

Changes in the secondary structure upon adsorption of β-casein (βCN) and of distinct parts of its sequence were investigated by far-ultra violet circular dichroism in order to find suggested relationships with foam and emulsion-forming and -stabilising properties of the same protein/peptides. A teflon/water interface was used as a model system for foam and emulsion interfaces. The maximum surface loads of βCN and its derived peptides were investigated. The main secondary structure element of all samples in solution was the unordered random coil, but upon adsorption ordered structure, especially α-helix, was induced. At lower pH more ordered structure was induced, just as at lower ionic strength. Apparently, both hydrophobic and hydrophilic groups influence the change of secondary structure induced at a hydrophobic interface. The results suggest that the hydrophobic C-terminal half of βCN accounted for the high maximum surface load on teflon, while the N-terminal half of βCN seemed to be responsible for the secondary structure induction upon adsorption. A relation between the maximum surface load and the foam-stabilising properties was found, but an influence of the secondary structure properties on the foam- and emulsion-forming and -stabilising properties was not observed.

This Chapter is based on:

Caessens, P.W.J.R.; De Jongh, H.H.J.; Norde, W.; Gruppen, H. The adsorption-induced secondary structure of β-casein and of distinct parts of its sequence in relation with foam and emulsion properties. *Biochim. Biophys. Acta*, **1998**, *in press*.

Introduction

Since milk proteins are often used in the food industry for their functional properties, such as the formation and stabilisation of foams and emulsions, they are well studied (see for reviews e.g. Swaisgood, 1982; Walstra and Jenness, 1984; Phillips et al., 1994; Wong et al., 1996). β-Casein (βCN), one of the major milk proteins, is an important protein involved in the formation and stabilisation of food emulsions (Dickinson, 1994). It is a strongly amphipathic molecule with a highly charged N-terminal part and a C-terminal part containing many hydrophobic side chains and only a few charged groups (Figure 1). At neutral pH most of the protein's net charge is located in the first 21 residues of the molecule (including 4 of the 5 phosphoserines [PSer], pK_1= 1.5 and pK_2= 6.4), while the net charge of the remainder of the molecule is nearly zero (Swaisgood, 1982). Several authors (e.g. Creamer et al., 1981; Graham et al., 1984) mentioned the low amount of secondary structure present in caseins. There are strong indications that in oil-in-water emulsions formed with βCN the N-terminal hydrophilic region of the adsorbed molecule projects into the aqueous phase while the hydrophobic remainder of the protein lies in direct contact with the oil surface (Dickinson, 1994; Horne, 1996).

In earlier Chapters we investigated the functional properties of βCN, and of several βCN-derived peptides (Chapters 2 and 3). Large differences were found in foam- and emulsion-forming and -stabilising properties as well as in surface-active properties between the several βCN peptides. Generally, the hydrophobic peptides possessed good foam-forming properties, whereas the strongly amphipathic peptides had emulsion-stabilising properties against coalescence.

A prerequisite for proteins and peptides to form and stabilise foams and emulsions is that they preferentially adsorb at the air/water and oil/water interface, respectively. Polystyrene latices have been used as a model system for emulsions (Dalgleish, 1990; Dalgleish and Leaver, 1991). In several studies the effect of adsorption on the secondary structure of proteins has been investigated using circular dichroism (CD) or Fourier transform infrared spectroscopy. It appeared that the secondary structure content of the proteins investigated either increased, decreased or did not change upon adsorption at hydrophobic surfaces; similar effects were observed at hydrophilic surfaces (Norde and Favier, 1992; Smith and Clark, 1992; Kondo and Urabe, 1995; Maste et al., 1996; 1997; Zoungrana et al., 1997; Fang and Dalgleish, 1997).

Several authors (Enser et al., 1990; Carey et al., 1994; Saito et al., 1995; Kang et al., 1996; Sheehan et al., 1998) suggested a relationship between the presence of secondary structure in synthetic peptides in solution, especially amphiphilic α-helices, and biophysical properties, such as surface activity and foam and emulsion-forming and -stabilising properties (further denoted as functional properties). This study focuses on the relationship between the secondary structure of βCN and some of its derived peptides both in aqueous solution and in the adsorbed state and their functional properties, as established in the previous Chapters (2 and 3). The βCN

derived peptides used were the hydrophilic peptide βCN[f 1-28], the amphipathic peptides βCN[f 1-105/107] and βCN[f 29-105/107] and the hydrophobic peptide βCN[f 106/108/114-209]. By using these peptides distinct parts of βCN could be investigated separately (Figure 1). The secondary structure of the protein/peptides was analysed using far-ultra violet CD. Teflon particles, which have a hydrophobic surface, were used to mimic foam and/or emulsion interfaces. The influence of pH and ionic strength on the secondary structure of the protein/peptides in these systems was investigated.

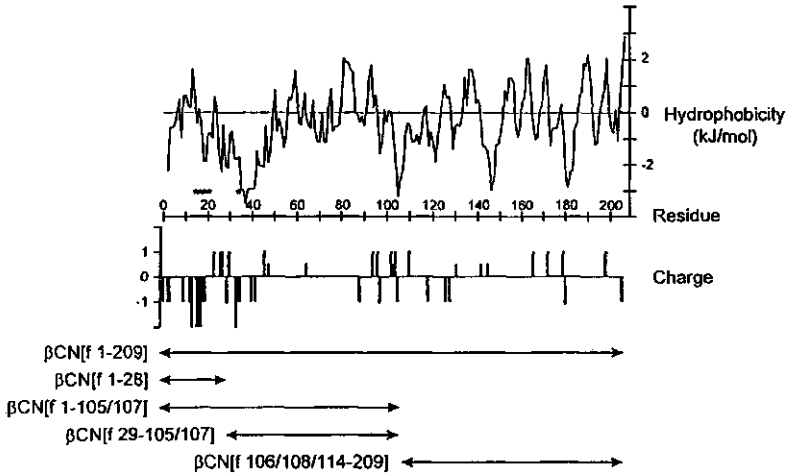

Figure 1. Schematic representation of the linear chain distribution of charged residues in βCN and the hydrophobicity map of βCN. The hydrophobicity map is constructed according to Kyte and Doolittle (1982) with a window of 5, and without taking the PSer-residues (approximate positions indicated with asterisks) into account. βCN-derived peptides used in this study are indicated.

Materials and methods

Materials

Bovine βCN (90% βCN based on weight, 95% βCN based on nitrogen, w/w) was purchased from Eurial (France), and contained mainly the genetic variants A^1 and A^2. Unless stated otherwise, all chemicals were of analytical grade, and were purchased from Merck (Germany). The sorbent used was a hydrophobic teflon latex (diameter $d \approx 215$ nm; density $\rho = 2.0$ g/cm^3) and was kindly donated by Du Pont de Nemours (Switserland). This material was further described by Maste et al. (1995; 1996), who showed that the latex dispersion was monodispers and free of contaminants. Before use, the teflon suspension was filtered over glass-wool, to remove aggregates that might be present, after which the concentration of the teflon suspension was determined (by measuring the dry weight %, w/w), and the surface-area per volume teflon suspension was calculated (m^2/mL).

Peptides

The peptides βCN[f 1-28], βCN[f 29-105/107] and βCN[f 1-105/107] were isolated as described earlier (Chapters 2 and 3), and were desalted using PD-10 columns (Pharmacia, Sweden), lyophilised and stored at 4°C. The peptide fraction βCN[f 106/108/114-209] was purified from the PEL-1 fraction described in Chapter 2 by preparative reversed-phase high-performance liquid-chromatography (RP-HPLC). The RP-HPLC equipment consisted of two M510 pumps (Waters Assoc., USA), a Gilson 231/401 injector (Gilson, USA), a Spectroflow 783 UV detector (Perkin Elmer, USA) and a Waters Type 680 automated gradient controller. The equipment was connected to Turbochrom data acquisition and processing software (Perkin-Elmer). A HiPore RP-318 column (21.5×250 mm I.D., Bio-Rad, USA) was used with a C_{18} cartridge (Bio-Rad) as a guard column. Solvent A (0.1% trifluoroacetic acid [TFA] in 10% aqueous acetonitrile, v/v) and solvent B (0.08% TFA in 90% aqueous acetonitrile, v/v) formed the eluent in the following linear gradient steps: in 1 min the flow was adjusted from 1.1 ml/min to the further applied flow rate of 8.8 ml/min at 37% B; from 37% B to 41% B over 38 min; to 70% B over 3 min followed by 5 min isocratic elution; to 37% B over 5 min; and finishing with 17 min equilibration at 37% B before the next run was started. For these preparative runs 900 mL of a 5 mg/mL protein solution (PEL-1 in solvent A) was injected onto the column at a flow rate of 1.1 ml/min. RP-HPLC was performed at 30°C. UV detection was at 220 nm. The two main peaks of the fraction, identified by mass spectrometry as βCN[f 106-209] and βCN[f 108/114-209] (Chapter 3), were collected and pooled, the acetonitrile was evaporated under vacuum at ambient temperature, and the remaining solution was freeze-dried before storage at 4°C. Since the experimental masses of the peptides were equal to the theoretical masses (maximal difference 0.02%), chemical modification of the peptides by RP-HPLC were ruled out.

Adsorption isotherms

All experiments described below were performed in sodium phosphate buffers at pH 4.0, 6.7 and 9.0 with an ionic strength (I) of 20 mM or 75 mM (adjusted with NaCl) at 20°C. In part, these conditions were chosen because in previous research (Chapters 2 and 3) the functional properties of the samples were determined at pH 6.7 and 4.0 and I = 75 mM, representative for milk and acid food products, respectively. The hydrophobic fraction βCN[f 106/108/114-209] is not soluble at these conditions. Therefore, studies at pH 9 (at which pH this fraction is soluble) were also included. To investigate the influence of the ionic strength, both I = 20 mM and 75 mM was used.

For all conditions, the maximum surface load (Γ in mg/m^2) of the hydrophobic teflon latices by βCN and the βCN peptides was established by solution depletion using a constant amount of surface area (1.26×10^{-2} m^2) and a varying protein concentration. The protein/peptide was allowed to adsorb at the teflon for at least 30 min (which was sufficiently long to attain adsorption saturation, Maste et al., 1995) after which the teflon/protein particles were centrifuged off (20 min at 14000 g;

Hermle Eppendorf centrifuge Z 200 M/H, Germany). The βCN(-peptides) concentration in the supernatant was determined by measuring the absorbance at 220 nm (Hitachi U-3000 Spectrophotometer, Japan). A calibration curve of the extinction at 220 nm of known concentrations (mg/mL) of each βCN-peptide was determined for all conditions used.

Conformational analysis

The same conditions as described above (20°C, pH 4.0, 6.7 and 9.0 and I = 20 and 75 mM) were used for the CD analysis of βCN(-peptides) both in solution and in the adsorbed state.

Far-ultra violet CD spectra were recorded as averages of 10 spectra on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan). Solutions of 0.1 mg/mL βCN (-peptides) in solution as well as dispersions of βCN(-peptides)-covered teflon particles were scanned using a 0.1 cm path length quartz cell. For the analysis of the adsorbed protein/peptide, the 0.1 mg/mL protein solution was incubated with defined amounts of teflon suspension required to obtain the maximum surface load (see above). The scan range was 260-190 nm, the scan speed was 100 nm/min, the data interval was 0.2 nm, the band width was 1.0 nm, the sensitivity was 50 mdeg and the response time was 0.125 s. No influence of the teflon in the far-ultra violet CD spectra of the protein free solvent was detected. Therefore, the CD spectra of the samples both in solution and in the adsorbed state were corrected by subtracting a protein and teflon free spectrum obtained under identical conditions. Noise reduction was applied according to the Jasco software. Variations in the secondary structure were estimated by comparing the ellipticity at 222 nm, and the CD zero-crossing point. Furthermore, spectral analysis to estimate the secondary structure content of the protein was performed using a non-linear regression procedure (De Jongh et al., 1994). Such a curve fitting procedure provides the relative contributions of the reference spectra that make up the best fit for the measured spectrum, and an estimation of the secondary structure can be calculated. Spectra were fitted from 240 to 190 or 195 nm (for sample with I = 20 and 75 mM, respectively) with 1 nm resolution using the reference spectra of poly-lysine in the α-helix, β-strand and random-coiled conformation (Greenfield and Fasman, 1969), and the spectrum of β-turn structures, extracted from spectra of 24 proteins with known X-ray structure (Chang et al.,1978).

Results

Adsorption isotherms of βCN and its peptides

Figure 2 shows the typical adsorption isotherms on the hydrophobic teflon of βCN and its derived peptides (determined by solution depletion at 20°C, pH 6.7 and I = 20 mM). The results demonstrate that the adsorption isotherms of βCN and the amphipathic βCN peptides reached plateau values within the concentration range

used (from 0 to 0.1 mg/mL). The isotherms for the amphipathic fractions βCN[f 1-105/107] and βCN[f 29-105/107] did not differ significantly. The maximum surface load of the intact protein was higher than that of the amphipathic peptides (approximately 3.0 and 2.0 mg/m^2, respectively). The adsorption isotherm of the hydrophilic peptide βCN[f 1-28] did not show a plateau value over the concentration range investigated. This peptide exhibited a lower affinity for the hydrophobic interface than βCN and the other peptides had. At pH 6.7 and I = 20 mM the hydrophobic fraction βCN[f 106/108/114-209] was insoluble, and the maximum surface load could, therefore, not be determined. At the other conditions, pH 4.0 and 9.0, I = 75 mM, the shape of the adsorption isotherms of each individual peptide was similar to that at pH 6.7 and I = 20 mM, but the absolute values of the maximum surface loads differed (no isotherms shown). Table 1 presents the maximum surface loads derived from the adsorption isotherms, determined at all pH's. The ionic strength had no significant influence on the plateau value of the adsorption isotherm (no further results shown). Generally, maximum surface loads increased with decreasing pH. Furthermore, at pH 9.0 the hydrophobic fraction (βCN[f 106/108/114-209]) had a higher maximum surface load than the amphipathic fractions, and it was similar to that of βCN.

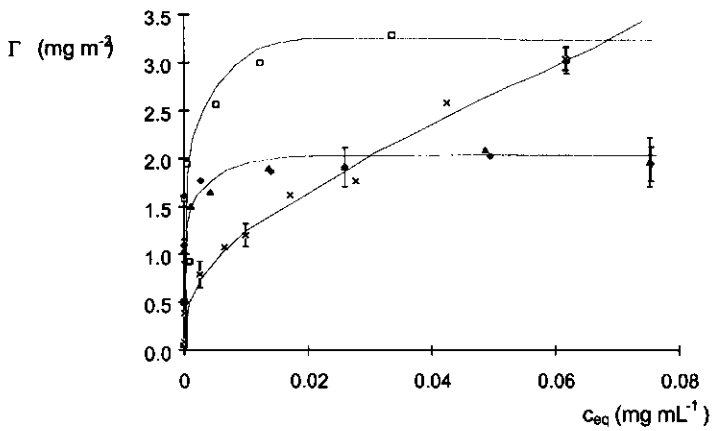

Figure 2. Adsorption isotherms of βCN (□), βCN[f 1-28] (×), βCN[f 1-105/107] (▲), βCN[f 29-105/107] (◆) on teflon at pH 6.7 and I = 20 mM. Surface load plotted against protein concentration in solution after adsorption.

Structural properties of βCN and its peptides

Several studies have shown that the degree of surface coverage of the sorbent surface influences the extent of changes in the secondary structure of proteins upon adsorption adsorption (Norde and Favier, 1992; Zougrana et al., 1997). Therefore, the CD measurements of the teflon-adsorbed protein/peptides were performed at

Table 1. Maximum surface load (Γ) on teflon used for CD experiments.

	Γ [mg/m²]		
Peptide	pH 4.0	pH 6.7	pH 9.0
βCN	n.d.	3.0	2.7
βCN[f 1-28]	2.2*	2.0*	1.7*
βCN[f 1-105/107]	2.2	2.0	1.7
βCN[f 29-105/107]	2.2	2.0	1.7
βCN[f 106/108/114-209]	n.d.	n.d.	2.7

* adsorption curve did not have a plateau value over the concentration range investigated

n.d. not determined, because sample was insoluble at these conditions

the saturation coverage shown in Table 1 only. At these conditions, maximally 10-20% (w/w) of the intact protein, the amphipathic peptides and hydrophobic peptides added was not adsorbed, and approximately 40% (w/w) of the hydrophilic peptide added was not in an adsorbed state.

Figure 3 shows typical CD spectra of βCN and one of the peptides (βCN[f 1-105/107]) at pH 6.7 and I = 20 mM, both in solution and in the teflon-adsorbed state. The spectra of the samples in solution did not have an ellipticity zero-crossing, and had a minimum around 200 nm indicating that both proteins had mainly an unordered structure (by curve-fitting procedures the estimated secondary structure was approximately 13% α-helix; 10% β-strand, 5% β-sheet, 72% random coil). All other samples measured in solution showed similar spectra (consisting of approximately 70-80% random coil) at all conditions tested (results not shown). The spectra of βCN and the βCN peptide in the adsorbed state showed a red-shift of the zero-crossing, when compared to the spectra in solution. Furthermore, the minimum

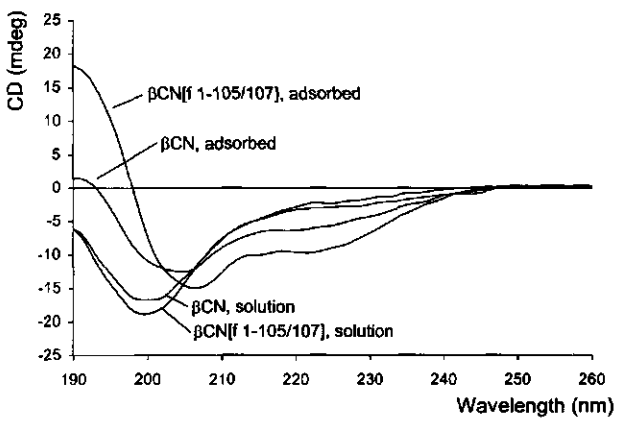

Figure 3. Far-ultra violet CD spectra of βCN and βCN[f 1-105/107] recorded at pH 6.7 and I = 20 mM free in solution and adsorbed at teflon.

around 200 nm had disappeared, and the spectra had two negative extremes around 208 nm and 222 nm, respectively, suggesting a more ordered secondary structure of the samples in the teflon-adsorbed state. Table 2 shows the molar ellipticity values at 222 nm as well as the ellipticity zero-crossing of the CD spectra of the protein and its derived peptides in the adsorbed state. The values at pH 6.7 show that βCN[f 1-28] has a zero-crossing at the lowest wavelength and the smallest value at 222 nm, which suggests that this peptide showed the lowest amount of secondary-structure induction, while the results for the strongly amphipathic peptide βCN[f 1-105/107] suggest the highest amount of structure-induction. Figure 4 shows the estimated percentages of random coil and α-helical structure for all samples measured at pH 6.7 and I = 20 mM. All fractions showed an increased amount of α-helix and a decreased amount of random coil upon adsorption. Generally, the induction of ordered structure upon teflon-adsorption was more pronounced for the amphipathic peptides than it was for βCN[f 1-28] and the intact molecule. When comparing the two amphipathic fractions, the structure induction upon adsorption was more prominent for the strongest amphipathic peptide, i.e. βCN[f 1-105/107] > βCN[f 29-105/107].

Table 2. Spectral evaluation of the CD spectra (molar ellipticity-values at 222 nm [Θ_{222}, deg cm^2/dmole][a] and extrapolated ellipticity zero-crossings [nm]) of teflon-adsorbed βCN and βCN peptides.

I = 20 mM	pH 4.0		pH 6.7		pH 9.0	
	Θ_{222} (×10^3)	zero-crossing	Θ_{222} (×10^3)	zero-crossing	Θ_{222} (×10^3)	zero-crossing
βCN	n.d.	n.d.	-7.0	193.1	-6.7	191.7
βCN[f 1-28]	-8.3	203.5	-5.4	192.7	-3.8	-
βCN[f 1-105/107]	-11.6	198.7	-11.3	197.7	-9.6	196.7
βCN[f 29-105/107]	˙	˙	-8.6	195.7	-7.4	194.1
βCN[f 106/108/114-209]	n.d.	n.d.	n.d.	n.d.	-6.2	192.9

[a] molar ellipticity of peptide bonds, calculated as described by Johnson Jr. (1996).
n.d. not determined because sample was insoluble at these conditions.
- no value found.
˙ sample/teflon-complex precipitated

Figure 5 shows the influence of pH (a) and ionic strength (b) on the CD spectrum of βCN[f 1-105/107]. The CD spectrum shows a red-shift of the zero-crossing with decreasing pH, which suggests an increasing α-helical induction (Figure 5a). The molar ellipticity values at 222 nm and the ellipticity zero-crossing of the CD spectra of the other peptides in Table 2 also suggest an increasing α-helical induction with decreasing pH. The values for molar ellipticity and the zero-crossing of the hydrophobic peptide βCN[f 106/108/114-209] are of the same magnitude as

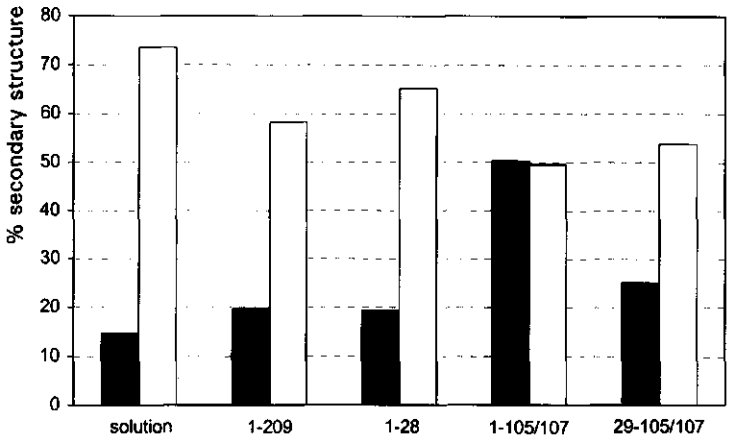

Figure 4. Analysis of far-ultra violet CD spectra showing the secondary structure content of βCN, βCN[f 1-28], βCN[f 1-105/107], βCN[f 29-105/107] in solution (average of all spectra in solution) and when adsorbed at teflon at pH 6.7 and I = 20 mM, presented as the percentages α-helical contribution (■) and random coil (□).

those for the intact protein, and this peptide has also a smaller content of secondary structure induction upon adsorption than the amphipathic peptides have. At pH 4 and after adsorption onto the teflon, the βCN[f 29-105/107]/teflon complex precipitated, and as a result the intensity of the CD spectrum of this sample decreased dramatically. At pH 4 this molecule contains little charged side chains, so that the electric repulsion of the teflon/peptide-complexes is probably too small to prevent aggregation. The CD spectra in Figure 5b show that the zero-crossing at I = 20 mM is at a higher wavelength than that at I = 75 mM, which suggests an increased amount of structure induction at lower I. Similar effects for the influence of the ionic strength were observed for the other samples tested (no results shown).

Discussion

A relationship between functional properties, such as surface activity, foam- and emulsion-forming and -stabilising properties, and the presence of secondary structure in synthetic peptides in solution was suggested in the literature (Enser et al., 1990; Carey et al., 1994; Saito et al., 1995; Kang et al., 1996; Sheehan et al., 1998). However, such a relationship has not been investigated for proteins used in food industry, e.g. βCN. To establish the structure-function relationship of βCN, the aim of the present study was to investigate the secondary structure of distinct parts of βCN (Figure 1), both in solution and in the adsorbed state, since it is known that the secondary structure of proteins can change upon adsorption (Norde and Favier, 1992; Smith and Clark, 1992; Kondo and Urabe, 1995; Maste et al., 1996; 1997;

Zoungrana et al., 1997; Fang and Dalgleish, 1997). The foam- and emulsion-forming and -stabilising properties of the same parts of the βCN-sequence (Figure 1) have already been investigated (Chapters 2 and 3).

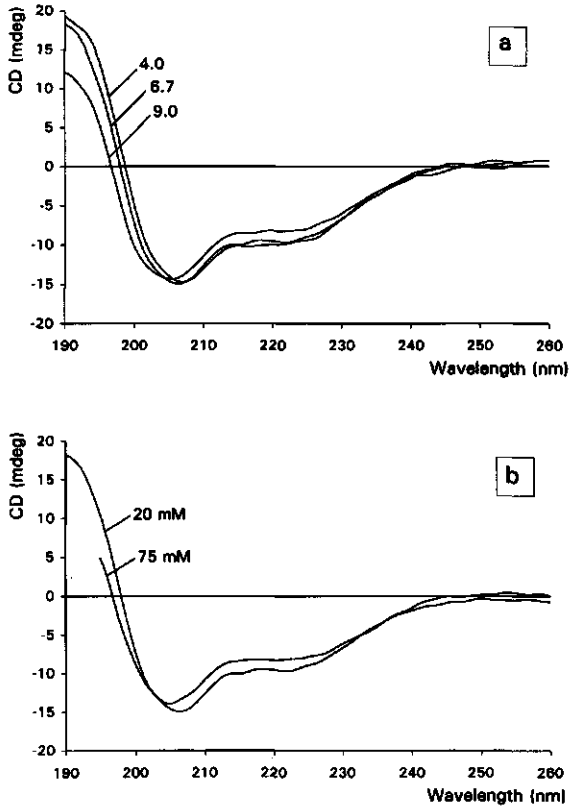

Figure 5. Far-ultra violet CD spectra of βCN[f 1-105/107] adsorbed at teflon recorded at pH 4.0, 6.7 and 9.0, I = 20 mM (a), and recorded at I = 20 mM and 75 mM, pH 6.7 (b).

The maximum surface loads of βCN on teflon latex as found in this study (Table 1) are in agreement (but slightly higher) than those reported for the same protein on several other surfaces, such as chemically hydrophobised glass, air/water and toluene/water interfaces (Brock and Enser, 1987; Graham and Phillips, 1979). Previous research (Smulders et al., 1998) showed that the maximum surface loads on soya oil-water emulsion-droplets of βCN and amphipathic βCN peptides at pH 6.7 and I = 75 mM are about 3.8 and 2.4 mg/m^2, respectively. For βCN and hydrophobic βCN peptides at pH 9.0 and I = 75 mM the maximum surface loads are approximately 3.2 mg/m^2. When the maximum adsorption values for the same peptides at the teflon interface obtained in this study (Table 1) are compared to those on the soy oil-water interface, the differences between the several peptides

are qualitatively similar in both systems. Therefore, these teflon particles may be used as a model for hydrophobic interfaces such as are present in emulsions (and foams).

An explanation for the increasing maximum surface load with decreasing pH (Table 1) could be that the negatively charged side chains (Asp, PSer and Glu) are less charged at lower pH (i.e. pH 4). Since the protein contains more negatively charged amino acids than positively charged amino acids (Arg, Lys and His), this probably allows the molecules to form a more closely packed layer at the interface, resulting in the higher maximum surface load. Similarly, the low content of charged side chains of the C-terminal-half of βCN could explain the high maximum surface loads of βCN and the hydrophobic βCN[f 106/108/114-209] at neutral pH and pH 9. However, diminishing the charge effect by using a higher ionic strength did not have an effect on the maximum surface load. Although the hydrophilic peptide βCN[f 1-28] had a lower affinity for the teflon interface, the surface load seems even to exceed that of βCN (Figure 2). Its adsorption isotherm showed no plateau value over the concentration range used, which could either indicate a multi-layer adsorption or a plateau-value reached at a higher peptide concentration.

Prediction of secondary structure of βCN based on the primary structure without taking the PSer-residues into account, performed according to Rost and Sander (Rost and Sander, 1993, 1994; Rost et al., 1994), showed that the intact molecule has some minor inclination for α-helix formation (segments 20-32, 40-48, and 189-193) which would result in a helical content of approximately 11%. This is in agreement with the CD results for βCN(-peptides) in solution found in this study (Figure 4), and with results found earlier for βCN (Creamer et al., 1981) and the βCN peptides βCN[f 1-28] and βCN[f 1-52] (Chaplin et al., 1988).

The α-helix content in the adsorbed state differed between the different βCN peptides. Although the presence of proline residues may influence the CD spectra (Bhatnagar and Gough, 1996), we believe that the small variance in proportion of proline residues between βCN, βCN[f 1-105/107], and βCN[f 29-105/107], 16.3%, 12.3% and 15.4%, respectively, does not account for the differences in α-helix induction found for these samples. The segment 20-32, possessing helix forming propensity (Rost and Sander, 1993, 1994; Rost et al., 1994), is not fully present in βCN[f 29-105/107], which could be a reason for its smaller α-helix induction compared to βCN[f 1-105/107]. The estimated α-helix content for βCN[f 1-28] was much lower than that for the amphipathic peptides. The structural predictions (Rost and Sander, 1993, 1994; Rost et al., 1994) for this peptide showed no propensity for α-helix induction, but some small segments had a probability for adopting stranded structures. Thus, the CD spectrum of βCN[f 1-28] in the adsorbed state (not shown) could also suggest β-strand induction (estimated percentage was about 15% β-strand); however, α-helix and β-sheet structures are hard to discriminate in CD spectra. The smaller content of structure induction of the hydrophilic βCN[f 1-28], compared to the amphipathic peptides, could not be explained by its low affinity to the teflon interface.

Lowering the pH from pH 9 to pH 4 increased the structure induction upon adsorption, suggesting an influence of the net charge of the molecule. Presumably, the formation of hydrogen bonds and the hydrophobic interactions are more distinct when less repulsion of strongly charged groups is present, resulting in a more ordered structure. The results obtained at different ionic strengths seem to contradict the influence of pH: less structure induction at higher ionic strength. Although from this result the charge of the side chains of the molecule seems to be an important factor for structure induction upon adsorption, no explanation for the discrepancy with the above pH effect observed can be given yet. Of course, both pH and ionic strength have an influence on the effective charge of the protein, but the way of influencing is different: lowering the pH results a lower amount of charged groups, while a higher ionic strength diminishes the effect of the charged groups. Besides the charge other effects (e.g. a steric effect of the phosphate groups concentrated in the N-terminal end) might have an impact on the structure induction as well.

Table 3 summarises some previously determined functional properties of βCN and its peptides determined at pH 6.7 as well as some structural properties determined in this study. The high maximum surface load found for βCN, and also for the hydrophobic peptide βCN [f 106/108/114-209] at pH 9, seems to correlate well with the good foam-stabilising properties; the maximum surface load of the amphipathic peptides, having worse foam-stabilising properties, is lower than that of βCN. It was demonstrated (Chapter 3) that βCN[f 1-105/107] had better emulsion-stabilising properties than βCN[f 29-105/107] had. The results obtained in this study showed that the stronger amphipathic peptide had a higher content of α-helices in the adsorbed state than the less amphipathic peptide, which might suggest a correlation with the emulsion-stabilising properties (Table 3). However, the structure induction in βCN is less than that in the amphipathic peptides, while the emulsion

Table 3. A summary of the emulsion and foam properties (as described earlier in Chapters 2 and 3) as well as some structural properties of βCN and several βCN-peptides.

	Γ[a]	α-helix[b]	Foam[c,d]		Emulsion[c,d]	
	[mg m^{-2}]	[%]	Formation	Stabilisation	Formation[e]	Stabilisation
βCN	3.0	20	++	+++	2.2	+++
βCN[f 1-28]	*	19	++	--	1.5	--
βCN[f 1-105/107]	2.2	50	++	-	1.5	++
βCN[f 29-105/107]	2.2	25	++	-	1.5	-
βCN[f 106/108/114-209]	n.d.	n.d.	++	+++	n.m.	aggregated

[a] determined at pH 6.7 and I = 20 and 75 mM

[b] estimated value calculated from CD spectra of adsorbed samples at pH 6.7 and I = 20 mM

[c] determined at pH 6.7 and I = 75 mM (Chapters 2 and 3)

[d] - and + indicate the extent of foam and/or emulsion formation and stabilisation

[e] average droplet-size (μm) immediately after homogenising

* the adsorption curve did not show a plateau value

n.d. not determined because sample was insoluble at these conditions

n.m. no droplet size could be measured because the emulsion was extremely aggregated

stability of the intact protein is superior to that of the peptides. Furthermore, the results found in this study also showed that at pH 4, when aggregated emulsions were formed with the peptides (Chapter 2), more α-helices were induced. Consequently, the relation of the structure-induction and functional properties in this system (βCN and βCN-derived peptides) does not point to the importance of secondary structure, which has previously been suggested for synthetic peptides (Enser et al., 1990; Carey et al., 1994; Saito et al., 1995; Kang et al., 1996; Sheehan et al., 1998).

In conclusion, our results show that ordered structure is induced in βCN(-peptides) after adsorption at teflon particles; the N-terminal amphipathic peptide (βCN[f 1-105/107]) shows more structure induction than the hydrophobic C-terminal peptide (βCN[f 106/108/114-209]). No relationship was found between the structure-induction of βCN(-peptides) and its foam- and emulsion-forming and -stabilising properties, but there appears to be a correlation between the surface-load and the foam-stabilising properties which seems to be governed by hydrophobic groups. The results further suggest that the N-terminal half of βCN (governed by the amphipathic peptide) seems to account for the structure induction of βCN, and that the hydrophobic C-terminal half of βCN is responsible for its high maximum surface-load.

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Chapter 5

Method for the isolation of bovine β-lactoglobulin from a cheese whey protein fraction and physicochemical characterisation of the purified product

Abstract

A method for preparing large quantities of native bovine β-lactoglobulin (βLg), suitable for research purposes, from a cheese whey protein fraction (WPF) was developed. The protein was isolated by combining a precipitation process (to precipitate all whey proteins, except βLg and caseinomacropeptide [CMP]), and a diafiltration process (to separate βLg, which remains in the retentate, from CMP, which is removed with the permeate). The yield of purified product from cheese WPF was approximately 60%.

The chemical composition and the physicochemical properties of the purified βLg were determined and compared with those of βLg purified according to Maté and Krochta (1994). The chemical composition of the two βLg samples was similar, both having a purity of 95% or higher (based on dry weight). The results of the physicochemical determinations indicated that, using the method described in this paper, βLg of at least the same quality as a product obtained according to Maté and Krochta (1994) can be produced in a reproducible and relatively easy manner.

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Introduction

β-Lactoglobulin (βLg), the major protein in bovine whey, is a well-described food protein (McKenzie, 1971; Swaisgood, 1982; Kinsella and Whitehead, 1989; Hambling et al., 1992). Native βLg is a globular protein with a monomer molecular weight of 18,300 Da (Hambling et al., 1992). The molecule possesses two disulphide bonds and one free thiol group, which exhibits an increased reactivity above pH 7 (Tanford et al., 1959; Dunnill and Green, 1965; Kinsella and Whitehead, 1989).

The βLg molecule can occur in different associated forms, depending on both temperature and pH (Kinsella and Whitehead, 1989). Native βLg occurs as a dimer in solution between pH 5.2 (isoelectric point) and 7.5. Between pH 3.5 and 5.2 βLg reversibly forms tetramers/octamers (Townend et al., 1960b; Townend and Timasheff, 1960), whereas below pH 3.5 it can dissociate into monomers (Townend et al., 1960a). It has been suggested that at temperatures between 30 and 55°C, the dimeric form of βLg dissociates to monomers. At even higher temperatures, unfolding of the molecule occurs, which results in increased activity and oxidation of the thiol group (Hambling et al., 1992). The βLg molecule is not denatured at acid pH (Aschaffenburg and Drewry, 1957; Kella and Kinsella, 1988).

βLg, like many other globular proteins, is surface-active and forms condensed films at the interface (Kinsella and Whitehead, 1989). A slight denaturation of the globular βLg molecule can have a great impact on its surface-active behaviour (Kinsella and Whitehead, 1989).

In the past, several methods have been described for the isolation of bovine βLg from milk (Larson and Jenness, 1955; Aschaffenburg and Drewry, 1957; Fox et al., 1967) and from cheese whey or cheese whey protein concentrate [WPC] (Pearce, 1983; Maté and Krochta, 1994; Kinekawa and Kitabatake, 1996; for a review with more references on the purification of βLg, see Hambling et al., 1992). In some purification methods different salt concentrations and pH adjustments are used to isolate βLg (Larson and Jenness, 1955; Aschaffenburg and Drewry, 1957; Maté and Krochta, 1994). Fox et al. (1967) exploited the solubility of βLg in trichloroacetic acid (TCA) to isolate the protein. Pearce (1983) has used the solubility of βLg in Cheddar cheese whey at pH 4.2 and 65°C to purify the protein, and Kinekawa and Kitabatake (1996) purified βLg from whey protein concentrate by a combination of pepsin treatment, salt precipitation and membrane filtration.

The objective of the present study was to prepare large amounts of bovine βLg from a cheese whey protein fraction (WPF) in a relatively simple manner. The βLg to be isolated was intended to be used for research purposes and, therefore, the protein had to be in its native form; hence denaturing conditions, such as elevated temperatures (Pearce, 1983) or pH values above pH 7, had to be avoided during the purification procedure. The method used to purify βLg, as described in this paper, is a combination of the methods of Fox et al. (1967) and Tanimoto et al. (1990). Fox et al. (1967) purified βLg from bovine milk by first precipitating the caseins at pH 4.6 and then precipitating all whey proteins except βLg by adding TCA to the acid whey. When

WPF prepared from cheese whey is used as a starting material, caseinomacropeptide (CMP) is also present in the resulting supernatant. Tanimoto et al. (1990) used a change in the apparent molecular size of CMP at different pH values for its isolation from cheese whey by ultrafiltration. While our work was in progress, the βLg purification method of Maté and Krochta (1994) based on βLg solubility at low pH and high salt concentrations was published. In the present paper we compare the chemical composition and some physicochemical characteristics of βLg purified by our method, βLg1, with data obtained with βLg purified according to Maté and Krochta (1994), βLg2.

Material and Methods

Materials and separation equipment

WPF (~ 86% protein), prepared according to De Wit and Bronts (1992), was obtained from DMV-International (the Netherlands). In the preparation process of this WPF the main whey proteins (βLg and α-lactalbumin) remained in their undenatured form. The reference protein (βLg2), prepared according to Maté and Krochta (1994) using Bipro, not containing CMP (Le Sueur Isolates, USA), as a starting material, was a gift of Dr. J.N. de Wit (DMV-International). A BGH Hermle ZK 630 centrifuge (I.K.S., the Netherlands) was used for centrifugation. Ultrafiltration equipment with 1.4 m^2 of membrane surface area and a 10 kDa molecular weight cut off [MWCO] (Romicon PM10, Koch, the Netherlands) was used for pilot plant-scale experiments. All chemicals were of analytical grade.

Isolation

The procedure followed to purify βLg from WPF is outlined in Figure 1. A 12% (w/v) aqueous solution of WPF (24 L) was divided into three parts which were each treated with equal volumes of 6% (w/v) TCA, resulting in final WPF and TCA concentrations of 6% and 3% (w/v), respectively. The precipitates formed were centrifuged off, and the total supernatant collected was divided into four batches of approximately 10 L each. Prior to diafiltration and ultrafiltration, these batches were diluted 10 times, and the pH was carefully adjusted to approximately 3.5 with 0.5 M NaOH under vigorous stirring to avoid local pH extremes. Diafiltration and ultrafiltration was performed using the indicated membrane system at an average pressure (P) of 1.3×10^5 N/m^2 and a DP of 0.7×10^5 N/m^2. During diafiltration, the pH of retentate 1 was kept between 3.5 and 3.8 by occasional addition of acetic acid (2 M). During ultrafiltration, the pH was not adjusted. The four resulting retentates 2 (Figure 1), with a pH of approximately 4, were mixed before lyophilisation. The purification procedure was monitored by reversed-phase high-performance liquid chromatography (RP-HPLC).

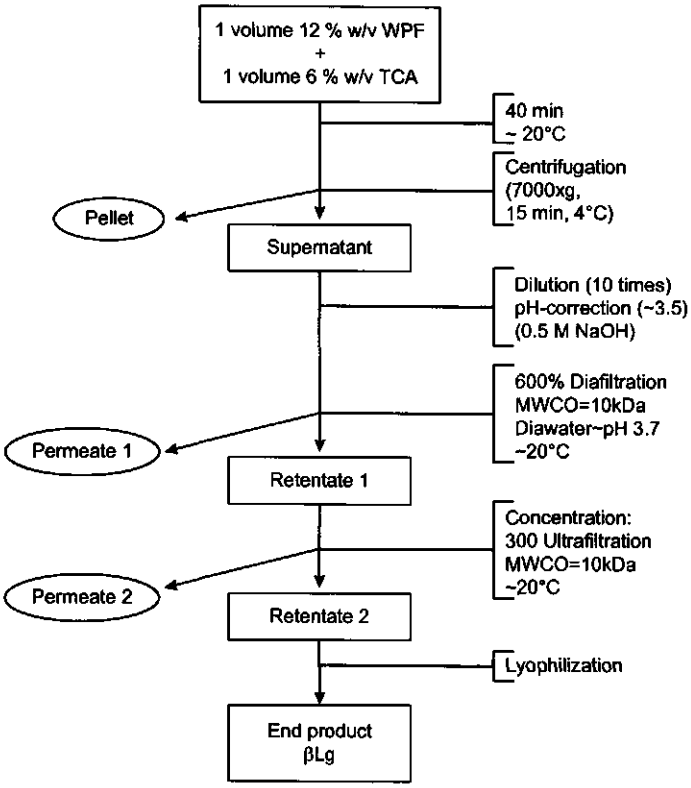

Figure 1. Procedure for the purification of βLg from a cheese WPF; MWCO: molecular weight cut off; 600% Diafiltration: six times the total volume of diawater is used for diafiltration; 300% Ultrafiltration: the total volume is three times reduced by ultrafiltration.

Characterisation

The protein content (N×6.38) was determined according to the Dumas principle (International Organisation for Standardisation, 1995) with a LECO FP2000 (LECO Instruments GmbH, Germany). The non-protein content (NPN) was analysed by a micro-Kjeldahl method after precipitation of high-molecular weight peptides and proteins with 12% TCA, essentially as described by Koops et al. (1975).

After extraction of the sample with heptane, the triglyceride content was determined in the organic phase by gas chromatography (GC) on a CPSIL5CB HT (10m×0.32mm) column, mainly according to Geeraert and Sandra (1985).

Lactose was determined by HPLC analysis on an HPX 87P column (85°C) with water as eluent and using refractive index detection.

Ash content was determined at 550°C. The water content was determined by the Karl Fischer-method using Reaquant®reagent (Verhoef and Barendrecht, 1977).

Organic components (such as TCA and acetic acid) were analysed by HPLC on an HPX 87H column with 0.1 M H_2SO_4 as eluent and UV detection (220 nm).

CMP was analysed by free-zone capillary electrophoresis [CE] (Van Riel and Olieman, 1995).

Amino acid analysis was performed on a 4151 Alpha Plus amino acid analyser (Pharmacia Biotech, Sweden) after hydrolysis of the samples with 6 M HCl in evacuated tubes at 110°C for 24, 48, 72 and 96 h. The amounts of Ser and Thr were corrected for hydrolytic losses by extrapolating their values to 0 h hydrolysis; values for Val and Ile after 96 h hydrolysis were used.

The βLg samples were also analysed by free-zone CE (De Jong et al., 1993), high-performance gel-permeation chromatography [HP-GPC] (TSK G2000SW$_{XL}$ column with 0.06 M potassium phosphate / 0.05 M sodium sulphate buffer (pH 6.5) as eluent (1.5 ml/min) and UV detection at 280 nm) and RP-HPLC (Visser and Slangen, 1992). The RP-HPLC equipment used was described in Visser et al. (1991).

In situ light-scattering measurements were performed to determine the aggregation behaviour of the samples at 3%, w/v based on dry weight, pH 7.0 and 68.5°C using a Malvern Autosizer IIC Submicron Particle Size Distribution Analyzer (Malvern Instruments Ltd, UK) according to Hoffmann et al. (1996).

Differential scanning calorimetry (DSC) experiments were carried out to measure the denaturation enthalpy and denaturation temperature at pH 6.5 (88 mg mL^{-1} and 10°C min^{-1}) using a DSC 7 (Perkin-Elmer).

Isoelectric focusing (IEF) was performed according to the instruction manual of Pharmacia, using a Phast gel IEF pH 4-6.5, a calibration kit (pH-range 2.5-6.5) and the Phast System (all from Pharmacia Biotech).

Results and Discussion

Isolation

βLg represented about 68% (expressed as % peak area in the RP-HPLC chromatograms) of the protein content of the WPF. Figure 2 shows the RP-HPLC chromatograms of the different fractions in the purification procedure. After TCA precipitation and centrifugation, the supernatant contained only βLg and CMP (86% and 14% of the total RP-HPLC peak areas, respectively). During diafiltration, the CMP was removed in the permeate and after 600% diafiltration, only a small amount of CMP remained in the retentate (approximately 4% of the total peak area in the RP-HPLC chromatogram). TCA precipitation and the diafiltration were performed in several batches, and the product composition (RP-HPLC) obtained with these process steps was very reproducible (results not shown). The yield of the purified product (βLg1) was approximately 1130 g (from 2880 g WPF), which is almost 60% (based on the βLg-content of the WPF).

Characterisation

The results of the compositional analyses of the purified βLg (βLg1) and the reference sample (βLg2) were compared (Table 1). Both samples had a protein

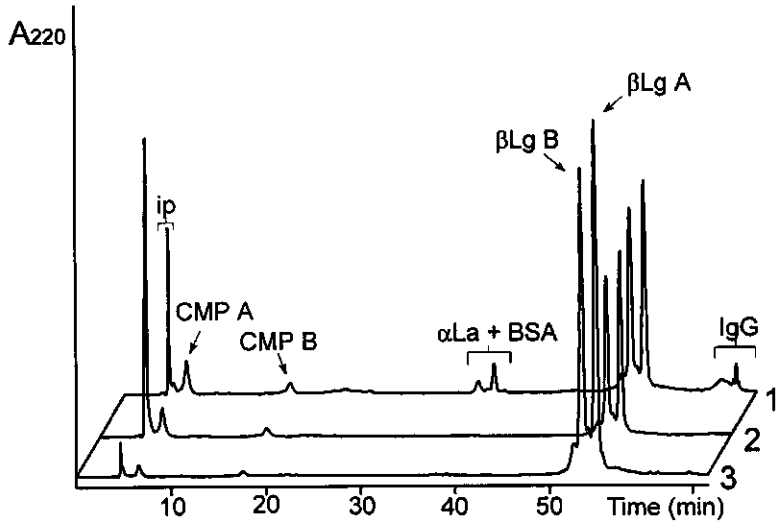

Figure 2. RP-HPLC chromatograms of βLg at different steps of purification from the cheese-whey protein fraction; 1: cheese-whey protein fraction; 2: trichloroacetic acid supernatant; 3: purified β-lactoglobulin after ultrafiltration (see Figure 1); CMP: caseinomacropeptide; αLa: α-lactalbumin; BSA: bovine serum albumin; IgG: immunoglobulin G, ip (injection peak): did not contain any protein material.

content of 95% or higher. βLg1 contained more NPN, which was probably caused by some remaining CMP (see Figures 2 and 3a). About 95% of the dry weight of βLg1 was βLg; the preparation contained some (trichloro)acetic acid which was possibly present as counter-ions (confirmed by HPLC). βLg2 contained approximately 2% ash, probably mainly NaCl resulting from the precipitation step (with NaCl and HCl) in the purification procedure, and resulting from the pH adjustment (with NaOH). In both samples, lactose and triglycerides were not detectable. The absence of fat from the βLg-samples is very important when the protein is used for studies on its foaming behaviour (Kinsella and Whitehead, 1989).

Table 2 shows the results of amino acid analysis. The amino acid compositions of

Table 1. Chemical composition of βLg1 and βLg2; values calculated as %, dry weight basis.

Sample	Protein [N×6.38]	NPN	Triglyceride	Lactose	Ash
βLg 1	94.7	1.1	n.d.	n.d.	< 0.2
βLg 2	98.6	0.4	n.d.	n.d.	2.1 ± 0.2

n.d. not detectable (< 0.01%)

the two samples were comparable and in agreement with data from the literature (Swaisgood, 1982).

HP-GPC analysis of the two samples were similar: the two chromatograms contained only insignificant differences in material with a high apparent molecular weight, possibly aggregated βLg molecules (βLg2 ≥ βLg1) and low molecular weight (βLg1≥βLg2; results not shown).

Table 2. Experimentally determined amino acid composition (mol/mol) of βLg1 and βLg2, and composition of βLg A and B according to the amino acid sequence (Swaisgood, 1982).

amino acid	βLg1	βLg2	βLg A (B)
Asx	15.7	15.3	16 (15)
Thr	10.0	8.3	8
Ser	8.0	7.1	7
Glx	26.0	24.7	25
Pro	11.3	9.9	8
Gly	3.9	3.8	3 (4)
Ala	14.8	14.3	4 (15)
Cys	2.9	2.7	2.5
Val	9.7	8.9	10 (9)
Met	4.1	4.0	4
Ile	10.4	9.6	10
Leu	20.5	20.6	22
Tyr	4.1	4.1	4
Phe	4.1	4.2	4
His	2.3	2.5	2
Lys	14.5	14.3	15
Arg	3.3	3.5	3

The composition of both βLg1 and βLg2, as shown in the RP-HPLC chromatogram (Figure 3a) was very similar: two main peaks representing βLg A and B, and some minor peaks (between the main peaks and just before βLg B). βLg2 had some absorbance at the same retention time as αLa and BSA whereas βLg1 contained some CMP.

The results of the CE analysis of the two βLg-samples (Figure 3b) were similar. Only the part where peaks were detectable in the electropherogram are shown; under the conditions used the two genetic variants of βLg were not separated. It appeared that the βLg1 peak had an indistinct shoulder. After the main βLg peak, both samples contained two small peaks which were not identified. Comparing the CE results with the RP-HPLC data, it can be observed that both techniques showed minor peaks near the main βLg peak. In laboratory-scale experiments, the influence of exposing βLg to high pH (approximately pH 11) on the RP-HPLC chromatogram was investigated. After this pH treatment, the normal βLg peaks had disappeared and new peaks with slightly lower and higher retention times appeared (results not shown). It could be that

the same (partly) denatured or aggregated products are represented by the minor peaks in the RP-HPLC chromatogram of Figure 3a as well as by the two small peaks observed in the CE-patterns (see Figure 3b). To obtain and preserve fully native βLg one should therefore avoid the use of strong alkali for pH adjustment, and in such cases use vigorous mixing to keep the local pH below about 7.

Heat treatment of βLg induces its aggregation and consequently the mass of the protein particles changes with time. The particle diameter increases rapidly from the beginning of the aggregation process, reaching a more or less constant value (Hoffmann et al., 1996). Bovine βLg samples of different origin show a similar and consistent behaviour, and higher amounts of salt result in an increase of particle size and scattering intensity (Hoffmann et al., 1996). The size of βLg particles during heat

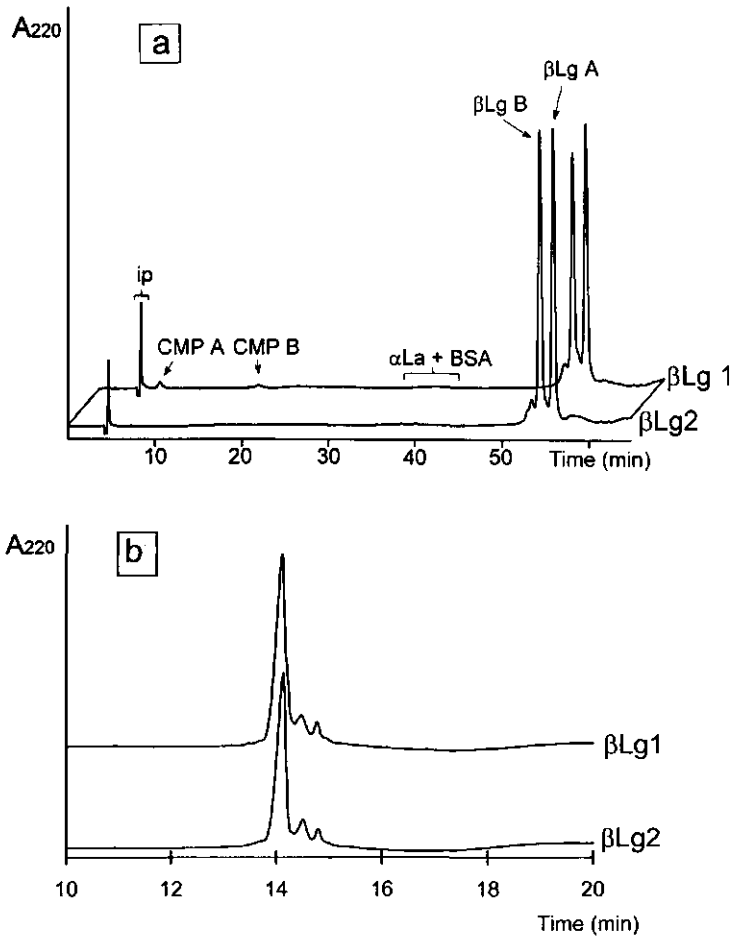

Figure 3 An analysis of βLg, purified according to the method described, βLg1, and prepared according to Maté and Krochta (1994), βLg2; (a) RP-HPLC; (b) CE; for abbreviations, see Figure 2.

treatment was monitored by light-scattering (Figure 4). It appeared that βLg1 exhibited an aggregation behaviour as expected: both the scattering intensity and the apparent particle size after heating were comparable with the results of Hoffmann et al. (1996). βLg2 had a rather high apparent particle size before heating, possibly due to the presence of traces of aggregated βLg in the sample. Both the particle size after heating and the scattering intensity of this sample were low compared to the values for βLg1 and to values reported in the literature (Hoffmann et al., 1996).

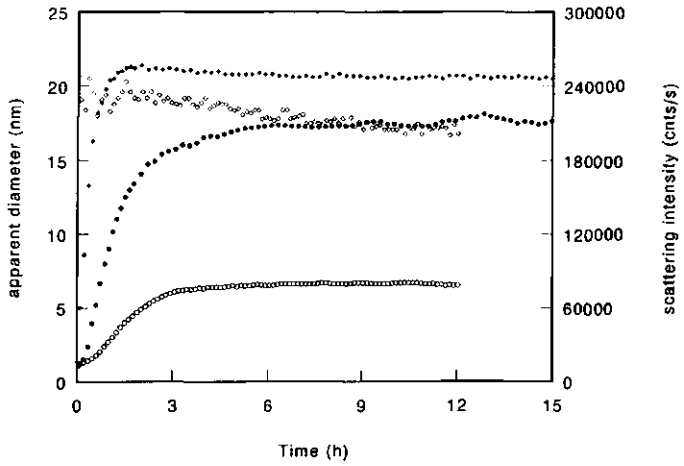

Figure 4 Aggregation behaviour of βLg1 and βLg2, 3% dry weight and pH 7, heated at 68.5°C, versus time as measured by *in situ* light scattering; for abbreviations, see Figure2; ● Scattering intensity βLg1,♦ Particle size βLg1;○ Scattering intensity βLg2,◇ Particle size βLg2.

From the results of the DSC experiments, it appeared that the denaturation temperature showed no difference between the two samples (T = 78.6°C), but βLg1 had a somewhat lower denaturation enthalpy than βLg2 (10.7±0.1 and 11.9±0.1 J/g protein, respectively; average values of triplicate measurements ± standard deviation), indicating that βLg2 was slightly more native than βLg1. The values obtained differed from literature data (De Wit et al., 1983), which is probably caused by differences in experimental conditions and equipment used. For this reason, only results of the two samples, measured under the same conditions could be compared.

The CE pattern of βLg1 showed an indistinct shoulder in the main peak (Figure 3b). However, IEF analysis showed no differences between the two βLg-samples (results not shown). The isoelectric points of βLg1 and βLg2 had the expected values (Swaisgood, 1982): 5.20 and 5.30 for the genetic variants A and B, respectively (results not shown).

The chemical composition of the two samples showed that only βLg1 contained a small amount of CMP (about 4%, w/w). The difference in starting material used for the

preparation of the two products was responsible for this difference in the end-products: βLg1 was purified from cheese WPF, containing CMP, while βLg2 was purified from Bipro, not containing CMP.

The physical properties of the two samples also showed some small differences. βLg2 seemed to show more aggregation of the βLg molecules (established by light-scattering experiments). The DSC results showed that βLg2 was at least of the same quality as βLg1 with respect to denaturation enthalpy. Some anomalous aggregation behaviour of βLg2, as established with light-scattering experiments, may be caused by traces of denatured and/or aggregated βLg, as discussed above. The TCA-precipitation method for the purification of βLg1 appeared to be a mild method. The results are consistent with those of Ebeler et al. (1990), who found no difference in secondary structure, measured by circular dichroism, between βLg isolated according to Aschaffenburg and Drewry (1957) and βLg prepared according to Fox et al. (1967), which suggests that the secondary structure of the molecule was not affected by precipitation with TCA.

With the present purification method, highly reproducible results can be obtained. Furthermore, the method can be performed on a large scale. The product yield in our process was about 60%. The remaining βLg was lost mainly in the TCA-precipitation and ultrafiltration steps. If the purification steps had been performed in one batch by using larger equipment, the loss of βLg would probably have been smaller.

During diafiltration, CMP was removed in the permeate, and with some simple adaptations of this process (for instance an extra diafiltration of the permeate to remove the TCA from the CMP) it can be purified separately within the same purification process, which could be a further advantage of this method.

Conclusions

The method to purify βLg from cheese WPF described in this paper proved to be very reproducible and easy to perform on a large scale. The preparation consisted of about 95% βLg (dry weight basis). Light scattering, DSC, IEF and HP-GPC indicated that our product is of at least the same overall quality as βLg prepared according to Maté and Krochta (1994). Our βLg appeared to be less aggregated than βLg purified according to Maté and Krochta (1994), as established with light-scattering experiments; this is essential for the study of heating processes, for example.

It can be concluded that the purification method described in this chapter produces native βLg of constant quality in large amounts, which is very important if the protein is to be used for research purposes.

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Chapter 6

β-Lactoglobulin hydrolysis. 1: Peptide composition and functional properties of hydrolysates obtained by the action of plasmin, trypsin and *Staphylococcus aureus* V8 protease

Abstract

β-Lactoglobulin (βLg) was hydrolysed by trypsin, plasmin and endoproteinase from *Staphylococcus aureus* V8 (*S.aur.*V8) to degrees of hydrolysis (DH) of 1, 2 and 4%. The several hydrolysates had different peptide compositions (determined by reversed-phase HPLC and gel-permeation chromatography [GPC]). GPC under non-denaturing, denaturing and denaturing + reducing conditions showed that the peptides formed were linked by hydrophobic interactions, by disulphide bonds, or were not linked at all. At very low protein concentration some differences in emulsion-forming properties were observed: only the plasmin hydrolysates could form emulsions with a uniform particle-size distribution. The emulsions formed with *S.aur.*V8 hydrolysates had poor emulsion-stabilising properties. Some hydrolysates showed increased foam-forming properties in comparison with the intact protein. All foams formed were stable. Overall, the plasmin hydrolysate (DH4) contained relatively much larger molecules and/or molecules with many hydrophobic groups; and many were disulphide-linked peptides. This hydrolysate also had the best functional properties.

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Introduction

In the food industry milk proteins are applied for their functional properties, such as foam- and emulsion-forming and -stabilising properties. Enzymatic hydrolysis can be used to alter the functional properties of proteins. It generally results in a decrease of molecular weight, an increase of ionizable groups and an increased exposure of hydrophobic groups (Panyam and Kilara, 1996). The choice of the enzyme used will determine which peptides will be formed because of differences in enzyme specificities. As a result, hydrolysates that have been formed by various enzymes may have different functionalities.

Already in 1974 it was reported that enzymatic hydrolysis of whey protein concentrate (WPC) with prolase, pronase, or pepsin resulted in improved foaming properties, but caused decreased emulsifying properties compared to the non-hydrolysed WPC. In addition, there seemed to exist an optimal degree of hydrolysis (DH) for improving functional properties (Kuehler and Stine, 1974). The influence of hydrolysis by several enzymes (Jost and Monti, 1982), to different DH's (Chobert et al., 1988) and the influence of hydrolysis and subsequent thermal treatment and/or fractionation of the WPC hydrolysates (Turgeon et al., 1991; Althouse et al., 1995; Lieske and Konrad, 1996) on functional properties of whey protein mixtures have been investigated ever since. However, the results from these studies are difficult to compare, because of the complexity of the protein mixture used and because different methods to determine the functional properties were used.

Approximately 50% of the whey proteins is composed of β-lactoglobulin (βLg). In its native form, βLg is a globular protein with a monomer molecular weight of approximately 18.3 kDa. βLg has 2 disulphide-bonds and a free thiol group; above pH 7.5 this free SH-group has increased reactivity. At neutral pH native βLg occurs as a dimer in solution and its associated form changes at different pH's (Swaisgood, 1982; Hambling et al., 1992). Native βLg is resistant to peptic and chymotryptic hydrolysis but the susceptibility of βLg to enzymatic hydrolysis often increases after protein denaturation (Reddy et al., 1988; Schmidt and Van Markwijk, 1993; Stapelfeldt et al., 1996). Research concerning the influence of enzymatic hydrolysis of purified βLg on the functional properties has been limited to tryptic hydrolysis. It was reported that βLg hydrolysates formed by the action of trypsin have altered interfacial properties (Turgeon et al., 1992), improved gelation properties (Chen et al., 1994) and decreased emulsion-stabilising properties (Agboola and Dalgleish, 1996) when compared to non-hydrolysed βLg.

The objective of the present study was to investigate the influence of enzymatic hydrolysis of purified βLg by different enzymes on the foam- and emulsion-forming and -stabilising properties (further denoted functional properties) in a systematic way. Therefore, βLg was hydrolysed by plasmin, trypsin and *Staphylococcus aureus* V8 protease (*S.aur.*V8) to DH 1, 2 and 4%. The peptide composition of the hydrolysates was analysed and the functional properties of the hydrolysates were determined and discussed in view of the peptide composition.

Materials and Methods

Materials

Bovine βLg (95% based on dry weight, w/w) was purified as described earlier (Chapter 5) and contained the genetic variants A and B in approximately equal amounts. Bovine plasmin (EC 3.4.21.7), trypsin (EC 3.4.21.4), aprotinin and soy bean trypsin inhibitor were obtained from Sigma (USA; article numbers P-7911, T-8642, A-6012 and T-9777, respectively). Endoproteinase Glu-C from *Staphylococcus aureus* V8 (*S.aur.*V8; EC 3.4.21.19) and *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Boehringer Mannheim (article numbers 791 156 and 874 485 , respectively). Unless stated otherwise, all other chemicals were of analytical grade and were purchased from Merck (Germany), Aldrich (Germany) or BDH (UK).

β-Lactoglobulin hydrolysis

A βLg solution (1% w/v; filtered through 0.22 μm filters [Schleicher and Schuell GmbH, Germany]) was hydrolysed by plasmin, trypsin and *S.aur.*V8 at 40°C and pH 8 to a DH of 1, 2 and 4% using a pH-stat method (Adler-Nissen, 1986). The plasmin hydrolysates are called PlDH1, PlDH2 and PlDH4, the trypsin hydrolysates TrDH1, TrDH2 and TrDH4 and the *S.aur.*V8 hydrolysates V8DH1, V8DH2 and V8DH4. The DH was calculated from the amount of base (0.1 M NaOH) used in the pH-stat technique (Adler-Nissen, 1986). Incubation times were between 10 min and 4 hours for the different hydrolysates. The enzyme/substrate ratios (E/S, on crude weight basis) used for the plasmin hydrolysis were 1/200 w/w, 1/100 w/w and 1/50 w/w for PlDH1, PlDH2 and PlDH4, respectively. Aprotinin was added to the incubates (ratio 1/200 v/v of a 10 trypsin inhibitor unit/mL solution) to terminate the hydrolyses. For trypsin hydrolysis the E/S ratios were 1/400 w/w for TrDH1 and TrDH2 and 1/200 w/w for TrDH4. Soy bean trypsin inhibitor (ratio 1/170 v/v of a 12 mg/mL solution) was added to stop these hydrolyses. For the *S.aur.*V8 hydrolysis the E/S ratios were 1/450 w/w for V8DH1 and V8DH2 and 1/250 w/w for V8DH4. The pH of the incubates was adjusted to pH 6 (0.5 M HCl) and TLCK was added (ratio 1/200 v/v of a 50 mg/mL solution) to terminate these hydrolyses.

After the hydrolyses were stopped, the hydrolysates were lyophilised and stored at 4°C before further analysis.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The RP-HPLC equipment used was described by Visser et al. (1991). Solvent A (0.1% trifluoroacetic acid [TFA] in 10% aqueous acetonitrile, v/v) and solvent B (0.08% TFA in 90% aqueous acetonitrile, v/v) formed the eluent in the following linear gradient steps: after 3 min isocratic elution at 100% A from 0% to 50% B over 50 min, then to 70% B over 5 min, after 5 min isocratic elution at 70% B to 100% A over 5 min, and finishing with 17 min equilibration at 100% A before the next run was started. The column temperature was 30°C and a flow rate of 0.8 mL/min was applied. Peak detection and quantitation was at 220 nm using Turbochrom data acquisition and

processing software (Perkin-Elmer, Germany). Generally, 50 mL of a 1 mg/mL sample solution was injected onto the column.

Gel-Permeation Chromatography (GPC)

The hydrolysates were analysed by GPC under non-denaturing conditions (0.125 M potassium-phosphate buffer containing 0.125 M sodium sulphate; pH 6.65) using a Superdex 75 column (Pharmacia, Sweden) on a SMART-system (Pharmacia). A flow rate of 40 mL/min was applied, 50 mL of a 1 mg/mL sample solution was injected and detection was at 220 and/or 280 nm. GPC was also performed under denaturing conditions (0.125 M potassium phosphate buffer containing 0.125 M sodium sulphate and 6 M urea; pH 6.65) and at denaturing + reducing conditions (0.125 M potassium phosphate buffer containing 0.125 M sodium sulphate, 6 M urea and 15 mM dithiothreitol [DTT]; pH 6.65) using a Superdex 75 column on an FPLC system (Pharmacia). The flow rate was 0.44 mL/min, 200 mL of a 1 mg/mL sample solution was injected and detection was at 280 nm. Prior to analysis under denaturing and denaturing + reducing conditions, the samples were incubated overnight at ambient temperature in the denaturing GPC buffer (the buffer for the denaturing + reducing conditions contained 0.13 M DTT). Fresh buffers were prepared each day. The performance of the columns was tested using a mixture of markers containing the proteins BSA [molecular weight (MW) = 67 kDa], chymotrypsin A [MW = 25 kDa], ribonuclease A [MW = 13.7 kDa] and vitamin C [MW = 176 Da]. All GPC analyses were performed at 20°C.

Functional properties

Foam and emulsion properties of the hydrolysates were tested in screening tests, which have been described earlier (Chapter 2). Conditions used were pH 6.7 (representative for neutral foods) and pH 4.0 (representative for acidic foods), ionic strength (*I*) of 75 mM and 20°C. Briefly, the screening tests were performed as follows. Emulsions (tricaprylin oil / water = 1/9; 0.2% and 0.05% w/v protein) were made using a laboratory high-pressure homogeniser and applying two passages at 60 bar. The emulsion-forming ability was determined immediately after homogenising by measuring the particle-size distribution (Malvern MasterSizerX, Malvern Instruments Limited, UK). The emulsion stability against coalescence was determined immediately after homogenising and again after 1 and 24 h by measuring the turbidity at 500 nm. The emulsions were also examined for the presence of aggregates and/or flocs using a light microscope. Foam-forming and -stabilising ability of a 0.01% (w/v) protein solution was tested using a whipping method as described earlier (Chapter 2) and the foam height was monitored for 1 hour.

Results

Peptide composition of the βLg hydrolysates

RP-HPLC was performed in order to characterise the peptide composition of the hydrolysates. Figure 1 shows the RP-HPLC chromatograms of the plasmin (a), trypsin (b) and *S.aur.*V8 (c) hydrolysates. From the figure it follows that the peptide composition of the hydrolysates produced by the three enzymes was different. Besides the peaks having the same retention time as intact βLg (which is present in all hydrolysates), the peaks in the chromatogram of V8DH4 are distributed over the whole chromatogram, while the chromatogram of TrDH4 shows mainly some peaks around 30 min and around 50 min retention time. The chromatogram of PlDH4 has mostly peaks with higher retention times (55-63 min). The hydrolysis of βLg by trypsin proceeded most rapidly, whereas βLg hydrolysis by plasmin had the slowest progress (e.g. DH1 was reached in approximately 5 min, 15 min and 30 min by trypsin, *S.aur.*V8 and plasmin, respectively). The peptide composition of the DH1, DH2 and DH4 hydrolysates produced by each enzyme did not change dramatically with increasing DH value; only the ratio of the peptides present differed. In each case, the peak of βLgA decreased more rapidly than that of βLgB, indicating that βLgA was degraded faster by enzymatic hydrolysis than βLgB. In all hydrolysates, peaks with the same retention time as intact βLg remained, making up approximately 33%, 36% and 18% of the total peak area in the RP-HPLC chromatograms of the PlDH4, TrDH4 and V8DH4, respectively.

Figure 2a shows the GPC chromatograms of the PlDH4, TrDH4 and V8DH4 hydrolysates obtained under non-denaturing conditions. From these results, it follows that PlDH4 contained the lowest amount of small peptides (i.e. elution volumes of > 1.7 mL). The main peak in the PlDH4 chromatogram had a similar retention time to that of the intact protein, suggesting that PlDH4 either contained a high amount of non-degraded βLg or degradation products having a similar retention time to that of βLg. V8DH4 contained the highest amount of smaller peptides and the smallest peak at a similar retention time as βLg.

The chromatograms in Figure 2a shows peaks after the included volume, indicating non-specific interaction of peptides with the column. Non-specific interactions could be avoided by analysing under denaturing conditions (with 6M urea added). Furthermore, by analysing in the presence of urea, hydrophobic interactions between peptides were prevented. Figure 2b shows the results obtained under denaturing conditions. The chromatogram of PlDH4 still had a main peak at the retention time of intact βLg (approximately 10 min), whereas the chromatogram of TrDH4 showed two distinct peaks with higher retention times (approximately 12 and 14 min). The V8DH4 hydrolysate contained a peptide composition that under denaturing conditions showed the overall smallest apparent MW and the lowest extinction at the retention time of βLg.

Figure 2c contains the GPC results obtained under denaturing + reducing conditions (after breaking both the intermolecular and intramolecular disulphide bonds

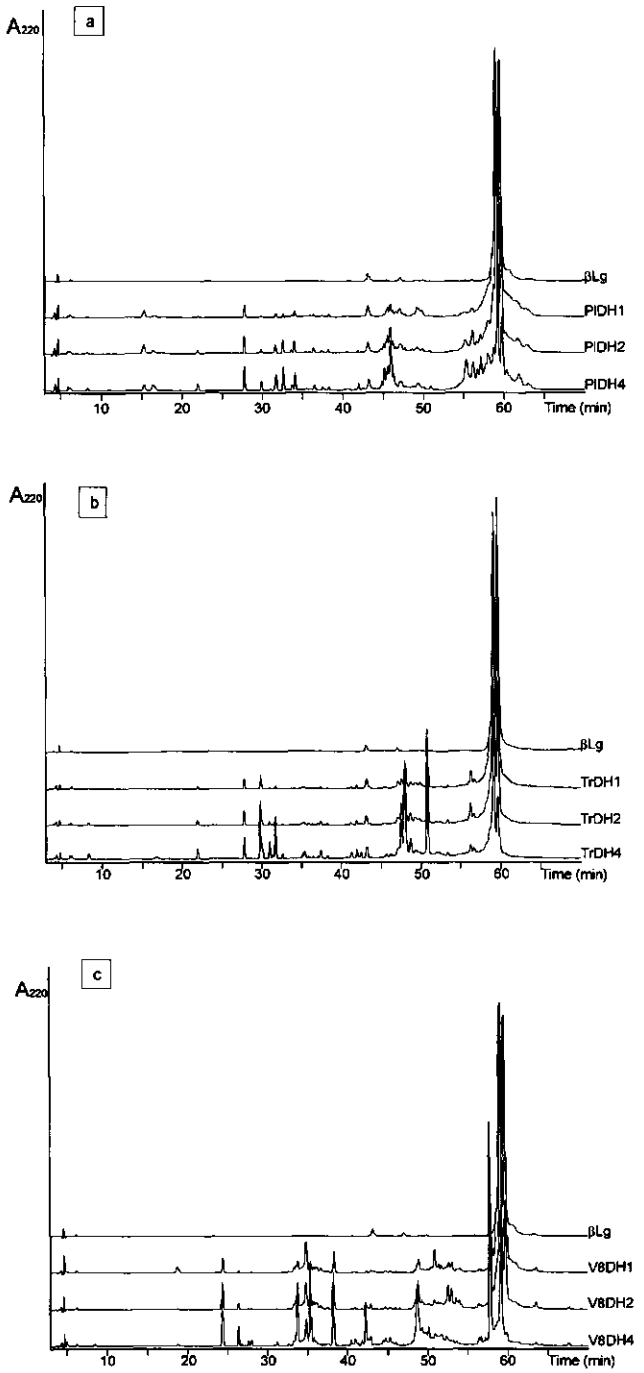

Figure 1. RP-HPLC chromatograms of the βLg hydrolysate fractions obtained by plasmin (a), trypsin (b) and *S.aur.*V8 hydrolysis (c); for abbreviations see text; for conditions used see Materials and Methods.

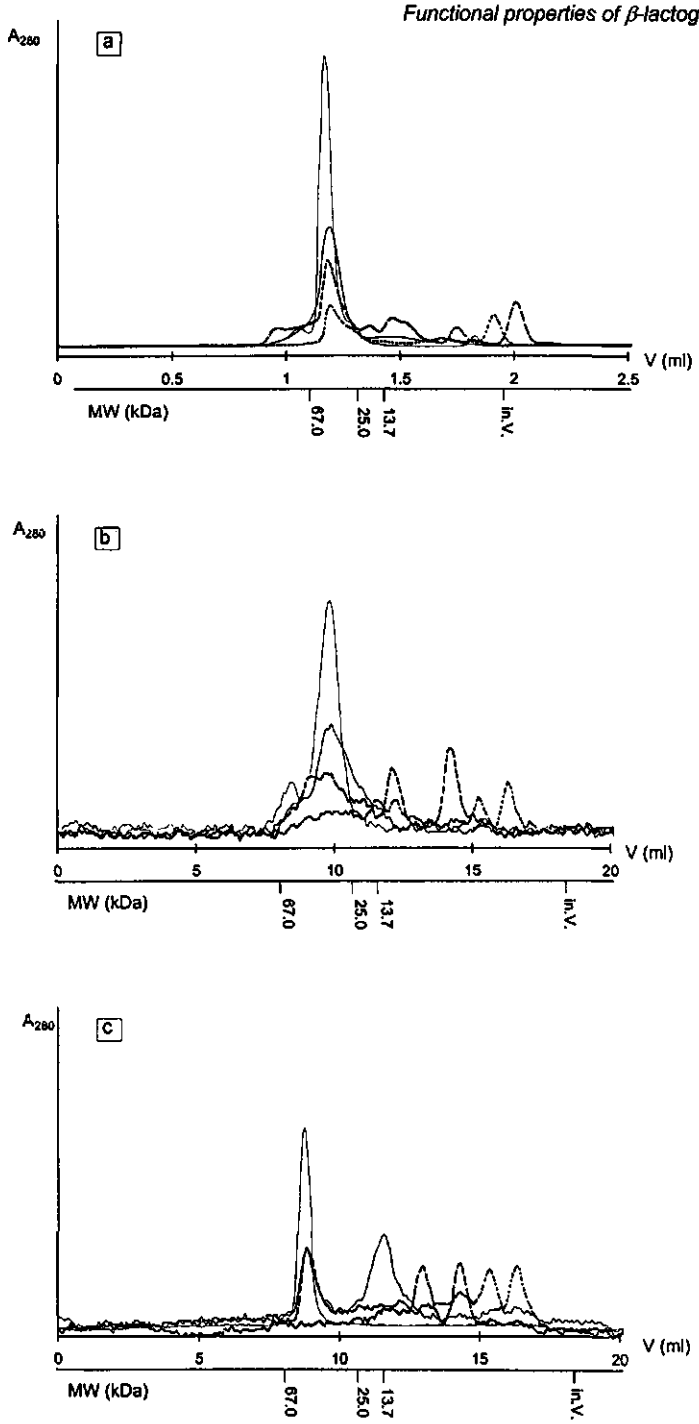

Figure 2. GPC chromatograms of PIDH4 (———), TrDH4 (· · · · ·) and V8DH4 (— — — ·) hydrolysate fractions and intact βLg (——) obtained under non-denaturing (a), denaturing (b) and denaturing + reducing (c) conditions; in.V. means included volume of the column, for further abbreviations see text.

in the hydrolysates). The chromatogram of PlDH4 showed a distribution of the peptides into two main peaks, while TrDH4 and especially V8DH4 had a broader distribution of the peptides. The latter hydrolysate contained no peak with a similar retention time to that of βLg, indicating that βLg was degraded completely. In the PlDH4 and TrDH4 chromatograms, peaks with the same retention time as βLg were present, making up approximately 29% and 28% of the total peak area.

The GPC chromatograms under non-denaturing, denaturing and denaturing + reducing conditions of the DH1, DH2 and DH4 hydrolysates of each enzyme showed a similar peptide composition; only the peak ratios differed (no further results shown).

Functional properties of the βLg hydrolysates

Figure 3 shows the results of the foam screening test at pH 6.7 (a) and pH 4.0 (b). At pH 6.7, the plasmin (Figure 3a-I) and *S.aur.*V8 (Figure 3a-III) hydrolysates formed more foam than intact βLg, whereas the amount of foam formed by the trypsin hydrolysates (Figure 3a-II) was about the same as that formed by βLg. In most cases less foam was formed at pH 4 than at pH 6.7 (compare Figure 3a and 3b). At the acidic pH, the trypsin (Figure 3b-II) and *S.aur.*V8 (Figure 3b-III) hydrolysates formed more foam than intact βLg, whereas the plasmin hydrolysate (Figure 3b-I) had similar foam-forming properties to βLg. At both pH's tested, the foam-stabilising properties of all hydrolysates were good: the foams were stable during 1 h and no coalescence was observed during the measurement.

Table 1 shows the results of the emulsion screening test at pH 6.7. At the highest concentration tested (0.2% w/v) the average particle size of the emulsion droplets formed by the hydrolysates and by βLg was similar; only the particle-size distribution (span) produced by the hydrolysates was smaller when compared to that produced by βLg. The stability (determined by measuring the turbidity at 500 nm) of these emulsions did not change during 24 h. Visually, the emulsions formed with V8DH4 and V8DH2 showed creaming after 1 and 24 h, respectively. Since no clear differences were observed at 0.2% w/v, emulsions were also made at a lower concentration (0.05% w/v). At this very low concentration, the emulsion-forming properties of the hydrolysates differed (Table 1). Only the plasmin hydrolysates (especially PlDH2 and PlDH4) formed emulsions with a uniform particle-size distribution, whereas the other hydrolysates formed emulsions containing a double peak in the particle-size distribution. Smaller and larger droplets were detected by microscopic analysis in the samples containing the double peak in the particle-size distribution (βLg, TrDH1, TrDH2, V8DH2 and V8DH4). The turbidity (500 nm) of the emulsions formed by V8DH4, V8DH2 and TrDH4 decreased during 24 h. Visually, the emulsion formed by V8DH4 already showed creaming after 1 h. Besides V8DH4, the hydrolysates V8DH2, TrDH4 and TrDH2 showed considerable creaming after 24 h, while βLg and the other hydrolysates formed emulsions with a minor amount of creaming after 24 h.

At pH 4 and 0.2% w/v, all hydrolysates and the intact βLg formed aggregated emulsions and the particle-size distribution could, therefore, not be measured with the Malvern MasterSizer X (no further results shown)

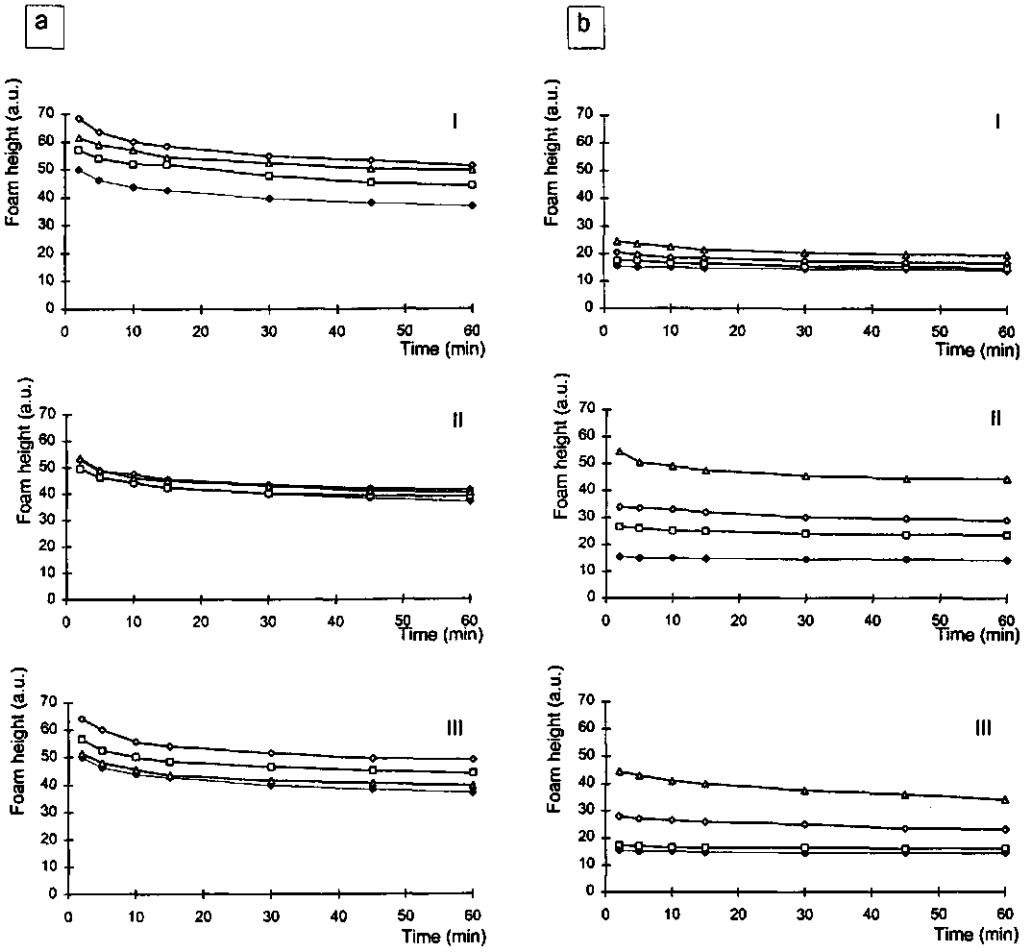

Figure 3. Foam height at pH 6.7 (a) and pH 4.0 (b) as produced with the βLg/plasmin (I), βLg/trypsin (II) and βLg/*S.aur.*V8 (III) hydrolysate fractions, as a function of time after whipping (means of duplicate measurements), intact βLg (◆), DH1 (□), DH2 (◇), DH4 (△): for abbreviations see text.

Discussion

Peptide composition

The RP-HPLC chromatograms (Figure 1) showed that the peptide composition of the hydrolysates formed by the three different enzymes differed, which was to be expected, since the enzymes used had different specificities. Plasmin and trypsin both hydrolyse Lys-X and Arg-X bonds (of which 15 and 3 are present in βLg, respectively), but plasmin is more selective and preferentially attacks Lys-X bonds (Bastian and

Table 1. Screening test results of emulsions made at pH 6.7 with βLg hydrolysates[a].

Sample	d_{32}[b] [μm]	span[b]	Stability[c]	d_{32}[b] [μm]	(1st/2nd peak)[e]	Stability[c]
Concentration	*0.2%*			*0.05%*		
βLg	1.7	2.6	++	2.7[d]	(2.5/10)	++
PIDH1	1.9	1.5	++	2.5[d]	(tails to 10)	++
PIDH2	1.8	1.5	++	2.3		+
PIDH4	1.7	1.6	++	2.5		+
TrDH1	1.9	1.9	++	2.7[d]	(2.5/10)	++
TrDH2	1.8	1.5	++	2.6[d]	(tails to 10)	+ cr
TrDH4	1.9	1.6	++	2.3		± cr
V8DH1	1.7	1.4	++	2.2		+
V8DH2	1.7	1.4	± cr	2.6[d]	(1.8/3.3)	± cr
V8DH4	1.8	1.2	- cr	3.2[d]	(1.7/6.2)	- cr

[a] For abbreviations used, see text.

[b] d_{32} is the average particle size of the emulsion droplets; the span indicates the distribution width

[c] +, ± and - indicate the extent of stability; cr means creaming.

[d] The measurement of the average particle-size showed a double peak (values of the separate peaks are shown in parentheses).

Brown, 1996). *S.aur.*V8 cleaves Glu-X and Asp-X bonds (of which 9 and 11(10) are present in βLgA(B), respectively). At pH 4 only Glu residues are hydrolysed, whereas at around pH 8 both Glu and Asp residues will be attacked (Drapeau, 1977). The PIDH4 fraction has many peaks with high retention times (55-60 min), indicating that the peaks contain large peptides and/or peptides with many hydrophobic groups. The TrDH4 fraction and particularly the V8DH4 fraction contain more peaks between 20 and 50 min, and probably contain smaller, less hydrophobic peptides than the PIDH4 fraction. Other authors also analysed βLg hydrolysates by RP-HPLC, e.g. tryptic hydrolysate fractions (Dalgalarrondo et al., 1990; Turgeon et al., 1992). The latter group also found peaks at the end of the chromatogram of the total hydrolysate (similar to our PIDH4 hydrolysate). It was suggested that these peaks might contain peptide aggregates, possibly induced by SH/SS exchange (Turgeon et al., 1992).

The RP-HPLC results (Figure 1) further show that βLgA is degraded faster by enzymatic hydrolysis than βLgB. This was already described in the literature (Schmidt and Van Markwijk, 1993), and the more flexible or less stable tertiary structure of βLgA was mentioned as a reason for its higher susceptibility to enzymatic degradation (Huang et al., 1994).

The amounts of βLg remaining in the DH4 hydrolysates determined by RP-HPLC were higher than those determined by GPC. This could indicate that peaks having the same RP-HPLC retention time as βLg not only contain intact βLg but also large peptides and/or peptides with many hydrophobic groups, which have different GPC-retention times. At the same DH, the βLg/*S.aur.*V8 hydrolysate overall has the lowest molecular sizes of the peptides compared to the βLg/plasmin and βLg/trypsin

hydrolysates, and contains the lowest amounts of intact βLg. As discussed above, peaks having the same retention time as βLg, can also be large peptides. Apparently, *S.aur.*V8 hydrolyses βLg in such a way that smaller peptides are released and that the larger peptides have a different chromatographic behaviour than intact βLg has.

The GPC results obtained under non-denaturing, denaturing and denaturing + reducing conditions showed that the peptides can be linked by hydrophobic interactions (in the case of the trypsin and *S.aur.*V8 hydrolysates, Figure 2b) as well as by disulphide bonds (most evident in the case of plasmin hydrolysates, Figure 2c). Turgeon et al. (1992) identified peptides present in a tryptic βLg hydrolysate, but could not assign the tryptic fragment βLg[f 102-124] to any of the peaks identified in the chromatogram. Since this part of the βLg sequence contains a disulphide group and a free thiol group, the possibility of SH/SS interactions between βLg peptides was mentioned (Turgeon et al., 1992). It is likely that similar SH/SS interactions occurred in the hydrolysates described in this study and that they were most evident in the plasmin hydrolysate.

Functional properties

Our results showed that the βLg hydrolysates had similar or increased functional properties compared to those of βLg. Increased functional properties of βLg after tryptic hydrolysis and subsequent fractionation of the hydrolysate have been observed earlier (Turgeon et al., 1992; Chen et al., 1994; Huang et al., 1996). In our case, the plasmin hydrolysates had the best foam- and emulsion-forming properties at pH 6.7. The functional properties of the plasmin hydrolysates decreased with increasing DH (no further results shown). Our results suggest an optimal DH for the functional properties, which has been suggested before (Kuehler and Stine, 1974; Arai and Fujimaki, 1991). However, it is generally excepted that besides the DH other factors, such as hydrophobicity, molecular size and amphipathicity, might determine the functional properties (Panyam and Kilara, 1996).

It should be noted that residual βLg, if present in the hydrolysates (Figure 1 and 2b), might account for (a minor) part of the functional properties determined. However, the improvement of the functionality of the hydrolysates compared to the intact protein had to be caused by the peptides. The reason for the improved emulsion-forming properties compared to those of βLg could be the higher concentration of molecules present in the hydrolysates. However, this cannot be the only reason, since the *S.aur.*V8 and trypsin hydrolysates did not have improved emulsion-forming properties. Other reasons (such as the influence of the hydrolysates on the surface tension at the air-water and oil-water interface, or the prevention of coalescence during the formation of the air bubbles and oil droplets) may be important for the formation as well (Walstra and Smulders, 1998) but have not been investigated for the hydrolysates in this study. The emulsion stability of the *S.aur.*V8 hydrolysates is poor. An explanation could be the low apparent MW of the peptides present (as analysed by GPC, Figure 2), since it is generally accepted that peptides must have a certain minimum MW to retain some functionality (Turgeon et al., 1992).

The main results found for the βLg hydrolysates formed by the action of the different enzymes can be summarised as follows:

residual amount of intact βLg:	*S.aur.*V8 < trypsin ~ plasmin
presence of hydrophobic groups:	plasmin > trypsin ~ *S.aur.*V8
presence of disulphide-linked peptides:	plasmin > trypsin ~ *S.aur.*V8
functional properties:	plasmin > trypsin > *S.aur.*V8

Limited hydrolysis by trypsin, plasmin and *S.aur.*V8 may improve the functional properties of βLg (the summarised functional properties mentioned above are mainly based on the increased emulsion-forming properties of the plasmin hydrolysates and the decreased emulsion-stabilising properties of the *S.aur.*V8 hydrolysates).

In conclusion, βLg hydrolysis by the action of plasmin resulted in a hydrolysate containing relatively large peptides with many hydrophobic groups, of which many were disulphide-linked peptides. This plasmin hydrolysate also had the best functional properties. In order to establish a structure-function relationship, further research has been focused on the fractionation of the PlDH4 hydrolysate, the functionality of the fractions, the identification of the peptides in the fractions and on the importance of the presence of the disulphide bonds (Chapter 7).

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Chapter 7

β-Lactoglobulin hydrolysis. 2: Peptide identification, SH/SS exchange and functional properties of hydrolysate fractions formed by the action of plasmin

Abstract

β-Lactoglobulin (βLg) was hydrolysed by plasmin to a degree of hydrolysis of 4%. The hydrolysate was fractionated by ion-exchange chromatography and subsequent hydrophobic-interaction chromatography. The βLg peptide fraction consisting of smaller peptides (mostly < 2 kDa) had poor foam- and emulsion-forming and -stabilising properties. Most of the βLg peptides were identified (in either the non-reduced or reduced form) by mass spectrometry on the basis of the known primary structure of the intact protein and the specificity of the enzyme. The peptides formed during βLg/plasmin-hydrolysis were (1) peptides lacking a cysteine residue, (2) peptides composed of a single amino acid chain containing intramolecular disulphide bonds, and (3) peptides composed of 2 amino acid chains linked by an intermolecular disulphide bond. It appeared that significant SH/SS-exchange had taken place during hydrolysis. Many of the peptides present in the peptide fraction that exhibited good functional properties, were disulphide linked fragments.

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Caessens, P.W.J.R.; Daamen, W.F.; Gruppen, H.; Visser, S.; Voragen, A.G.J. β-Lactoglobulin hydrolysis. 2: Peptide identification, SH/SS exchange, and functional properties of hydrolysate fractions formed by the action of plasmin.

Introduction

The formation and stabilisation of foam and emulsions by β-lactoglobulin (βLg)/plasmin, βLg/trypsin and βLg/*Staphylococcus aureus* V8 protease hydrolysates were described in the first part of this study (Chapter 6). The βLg/plasmin hydrolysate with a degree of hydrolysis of 4% (DH4) had good functional properties at pH 6.7. Reversed-phase HPLC showed that this βLg/plasmin DH4 hydrolysate contained peptides eluting in the second part of the chromatogram, indicating that the peptide material present was hydrophobic and/or had a rather high molecular weight. Furthermore, this hydrolysate contained many peptides that were linked by disulphide bonds as determined by comparative gel permeation chromatography under non-denaturing, denaturing and denaturing + reducing conditions (Chapter 6).

βLg has a monomer molecular mass of about 18.3 kDa (βLgA = 18362 Da and βLgB = 18277 Da). The protein has 2 disulphide bonds, between residues 106 and 119, and between residues 66 and 160, and a free thiol group at residue 121 (Swaisgood, 1982; Hambling et al., 1992). In the native protein, the free thiol group is buried in the interior of the molecule but at higher pH (i.e. above pH 7.5; Tanford et al., 1959) and/or elevated temperatures (Iametti et al., 1996), the protein undergoes conformational changes resulting in an increased reactivity of the thiol group. Thiol/disulphide exchange plays a role in the heat-induced aggregation of βLg (Roefs and De Kruif, 1994; Sawyer et al., 1994). Chen et al. (1994) found that tryptic βLg hydrolysates had a lower gel point and gelled more rapidly than native βLg. Of course, after βLg hydrolysis the free thiol group is more exposed and one could hypothesise that as a result, βLg hydrolysis might also initiate SH/SS reshuffling which would subsequently induce the aggregation and gelling behaviour described above.

Several authors have identified peptides produced by βLg/trypsin hydrolysis. Some of these peptides were disulphide linked, such as βLg[f 41-69] -S-S- [f 149-162] (Dalgalarrondo et al., 1990), βLg[f 61-69/70] -S-S- [f 149-162] (Turgeon et al., 1992), βLg[f 41-100] -S-S- [f 149-162] (Chen et al., 1993) and βLg[41-70] -S-S- [f 149-162] (Otte et al., 1997a). The disulphide bond present in these fragments was originally present in the parent protein (i.e. between residues 66 and 160). No disulphide-linked peptides have been identified in βLg hydrolysates formed by other enzymes, such as bromelain, papain, pepsin, or endoproteinase Arg-C (Otte et al., 1997a).

Turgeon et al. (1992) suggested the formation of new disulphide bonds during hydrolysis of βLg at pH 8 due to the increased reactivity of the free thiol group. Recently, strong indications of the occurrence of a newly formed disulphide bond in a βLg peptide have been published (Maynard et al., 1998). The first part of our study (Chapter 6) showed that the βLg/plasmin DH4 hydrolysate had good functional properties. In this part of the study, the βLg/plasmin DH4 hydrolysate was fractionated, the functional properties of the fractions were determined, and the

peptides present therein were identified to investigate the occurrence and importance of the SH/SS exchange between the βLg peptides. This allows a discussion of the functional properties in relation to the peptide composition, aiming at the establishing of a structure-function relationship.

Materials and Methods

Materials

Bovine βLg (95% based on dry weight, w/w) was purified as described earlier (Chapter 5) and was a mixture of the genetic variants A and B. Bovine plasmin (EC 3.4.21.7) and aprotinin were obtained from Sigma (USA; article numbers P-7911 and A-6012, respectively). Unless stated otherwise, all other chemicals were of analytical grade and were purchased from Sigma (USA), Merck (Germany), Aldrich (Germany) or BDH (UK).

β-Lactoglobulin hydrolysis and fractionation of the hydrolysate

βLg was hydrolysed by plasmin at pH 8.0 and 40°C to a DH of 4% as described earlier (Chapter 6). After incubation, the reaction was inhibited by aprotinin (ratio 1/200 v/v of a 10 trypsin inhibitor unit/mL solution); the hydrolysate was lyophilised and stored at 4°C prior to analysis or fractionation. The latter was performed by ion-exchange chromatography (IEC) and subsequent hydrophobic interaction chromatography (HIC). Figure 1 shows a brief outline of this fractionation.

Preparative IEC was performed on an ÄKTA-explorer, controlled by a UNICORN-control system (Pharmacia, Sweden), using a SourceQ column (280 mL bed volume; Pharmacia) at 20°C. Solvent A (20 mM bis-tris/HCl buffer, pH 6.0) and solvent B (20 mM bis-tris/HCl buffer containing 0.5 M NaCl, pH 6.0) formed the eluent in the

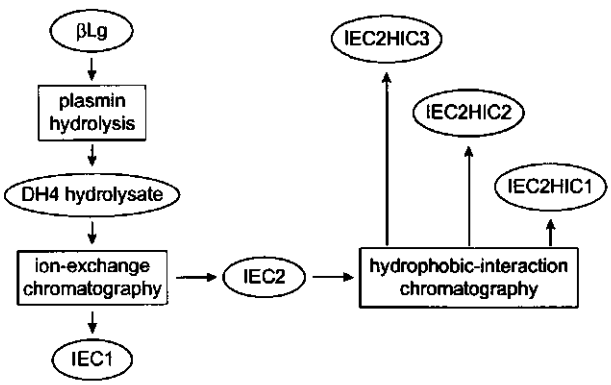

Figure 1. Outline of the βLg hydrolysate fractionation; for abbreviations used see text.

following linear gradient steps: 2 min sample injection (25 mL/min); 5 min isocratic elution at 100% A, over 2.5 min to 100% B followed by 2 min isocratic elution at 100% B, over 1 min to 100% A and finishing with 7.5 min equilibration of the column at 100% A before the next run was started. The column was loaded with maximally 4 mg per mL bed volume. Except stated otherwise, a flow rate of 44 mL/min was applied and detection was at 220 and 280 nm. Appropriate IEC fractions were pooled, desalted by ultrafiltration (OMEGA membrane MWCO of 1 kDa for the IEC1 fraction (Figure 1) and 3 kDa for the IEC2 fraction (Figure 2), Pall Filtron Corp., USA), lyophilised and stored at 4°C prior to analysis.

Preparative HIC was performed on the ÄKTA-explorer described, using a Phenyl Sepharose High Performance column (150 mL bed volume, Pharmacia) at 20°C. The sample to be purified, i.e. fraction IEC2 (Figure 1), was collected from the preparative IEC runs; ammonium sulphate was added to 1 M and the pH was adjusted to pH 7.0. The protein load of the HIC column was approximately 1 mg per mL bed volume. Solvent A (50 mM sodium phosphate buffer containing 1.7 M ammonium sulphate, pH 7.0) and solvent B (50 mM sodium phosphate buffer, pH 7.0) formed the eluent in the following linear gradient steps: 20 min sample injection (10 mL/min), 5 min isocratic elution at 35% B, over 1 min to 50% B, after 10 min isocratic elution over 10 min to 100% B, after 15 min isocratic elution over 2 min to 100% A, after 8 min isocratic elution over 2 min to 35% B and 12 min equilibration of the column before the next run was started. Except when stated otherwise, a flow rate of 21 mL/min was applied and detection was at 220 and 280 nm. Appropriate HIC fractions were pooled, desalted by ultrafiltration (OMEGA membrane 5 kDa MWCO), lyophilised and stored at 4°C prior to analysis.

Reversed-Phase High-Performance Liquid-Chromatography (RP-HPLC)

The RP-HPLC equipment used was described by Visser et al. (1991). To analyse the peptide composition of the different βLg peptide fractions (Figure 1), the same gradient as described before (Chapter 6) was used (denoted RP1), and generally 50 mL of a 1 mg/mL peptide solution was injected onto the column for these analytical runs. Gradient RP1 was also used for the semi-preparative RP-HPLC of smaller, more hydrophilic peptides present in IEC1 (Figure 1) and 150 mL of a 1 mg/mL peptide solution was injected onto the column. Gradient RP-2, used for the semi-preparative RP-HPLC of larger peptides containing many hydrophobic groups, present in IEC2HIC3 (Figure 1) was formed by solvent A [0.1% trifluoroacetic acid (TFA) in 10% aqueous acetonitrile, v/v] and solvent B [0.08% TFA in 90% aqueous acetonitrile, v/v] in the following linear gradient steps: from 18% B to 20% B over 2 min, to 54% B over 104 min, to 70% B over 4 min; after 5 min to 18% B over 5 min and finishing with 17 min isocratic elution at 18% B before the next run was started. A flow rate of 0.8 mL/min was applied. The column temperature was 30°C, and 150 mL of a 5 mg/mL peptide solution was injected onto the column. Peak detection and quantitation was at 220 nm using Turbochrom data acquisition and processing software (Perkin-Elmer, Germany).

Peptide identification

The peptides collected by semi-preparative RP-HPLC using gradient RP1 were analysed by electrospray-ionization mass-spectroscopy (ESI-MS) on a Quattro II triple quadrupole instrument (Micromass, UK) as described before (Chapter 3). Peptide identification was obtained from the molecular mass determined combined with the sequence data of intact βLg (Hambling et al., 1992) and the known specificity of plasmin (Arg-X and Lys-X; Bastian and Brown, 1996). The peptides collected by semi-preparative RP-HPLC using gradient RP2 were analysed under non-reducing conditions by ESI-MS as described before (Chapter 3), and under both non-reducing and reducing conditions by matrix-assisted laser-desorption/ionisation time-of-flight MS (MALDI-TOF MS). The latter was performed on a Voyager-DE™RP (PerSeptive Biosystems, USA) in the linear mode, controlled by Voyager RP software. The samples were dissolved in a 20 mM tris/HCl buffer (pH 7) with and without 20 mM dithiothreitol for the reducing and non-reducing conditions (reduction for approximately 30 min at ambient temperature), respectively; 1 mL of this peptide solution was mixed with 9 mL matrix solution. The matrix solution consisted of sinapinic acid (10-15 mg/mL 3,5 dimethoxy-4-hydroxy cinnamic acid in acetonitrile : 3% w/v TFA : water = 3 : 1 : 6). The final mixture (2 mL) was loaded on a welled plate and was allowed to dry in air. All samples were applied in duplicate. At least two different spots were analysed from each well and the masses obtained (representing protonated molecules, i.e. actual mass + 1 H^+) were averaged. External calibration was performed, under the same conditions as used for the analysis, using bovine insulin (5734.6 Da and 2867.8 Da for single and double protonated molecules, respectively), thioredoxin from *E.coli* (11674.5 Da and 5837.7 Da for single and double protonated molecules, respectively) and horse apomyoglobin (16952.6 Da and 8476.8 Da for single and double protonated molecules, respectively). Horse heart cytochrome *c* (single protonated mass 12361.5 Da) was used as an external reference protein.

Functional properties

Foam and emulsion properties of the hydrolysates were tested (at pH 6.7, ionic strength of 75 mM and 20°C) in screening tests, which have been described earlier (Chapters 2 and 6). The protein/peptide concentration used for the emulsion screening test was 0.05 % (w/v) and for the foam screening test 0.005% (w/v). The foam screening test was also performed under reducing conditions. Therefore, the foam test was performed in the presence of 30 mM DTT with preceding overnight incubation of the sample at ambient temperature.

Results

Fractionation of the βLg/plasmin DH4 hydrolysate

The DH4 hydrolysate could be separated by IEC into an unbound and a bound fraction, IEC1 and IEC2, respectively (Figure 2a). Fractions IEC1 and IEC2 made up

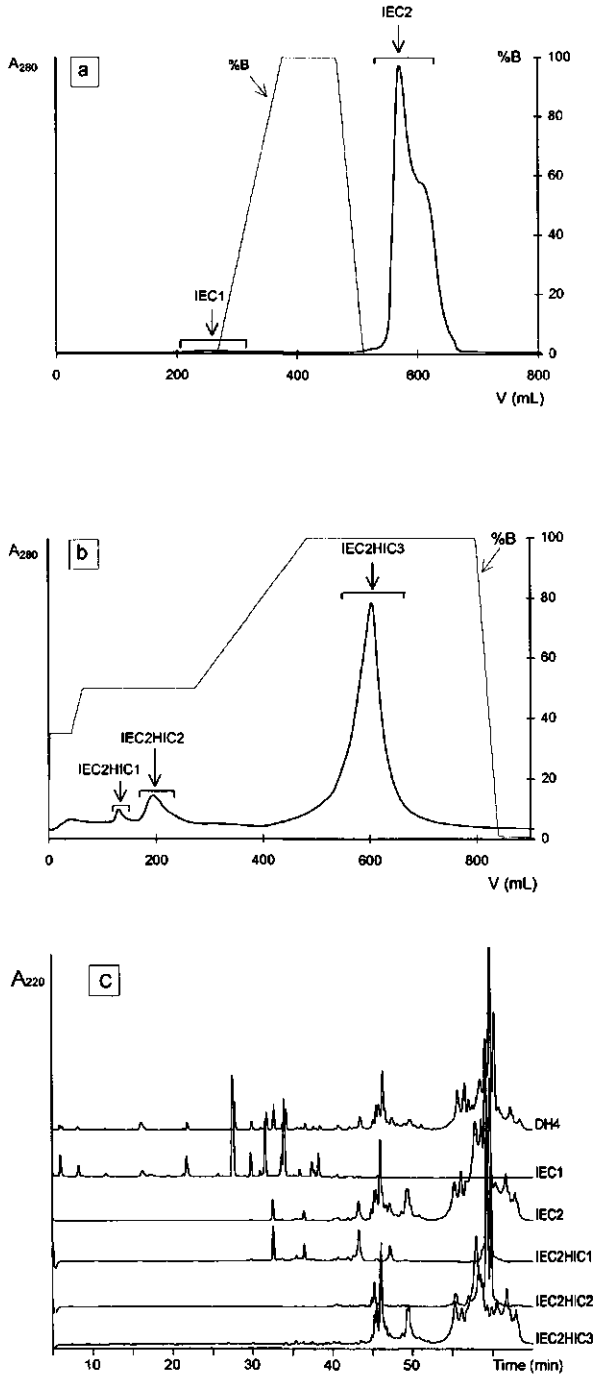

Figure 2. IEC chromatogram of the βLg /plasmin DH4 hydrolysate (a); HIC chromatogram of IEC2 (b); RP-HPLC chromatograms of the peptide fractions obtained by IEC and HIC (c); for abbreviations see text; RP-HPLC gradient RP1.

about 1% and 99% of the total peak area in the IEC chromatogram, respectively. Fraction IEC2 was further separated by HIC (Figure 2b), resulting in three peaks denoted IEC2HIC1, IEC2HIC2 and the major peak IEC2HIC3, making up approximately 2%, 10% and 88% of the total peak area in the HIC chromatogram, respectively. Figure 2c shows the RP-HPLC chromatograms of the several fractions obtained by IEC and HIC. Fraction IEC1 contained material eluting in the first part of the chromatogram, representing more hydrophilic and/or smaller peptides. The DH4 hydrolysate and IEC2 still contained some intact βLg, which was removed from IEC2 with the fractions IEC2HIC1 and IEC2HIC2. Fraction IEC2HIC3 did not contain detectable amounts of βLg (as analysed by RP-HPLC using an adjusted gradient, results not shown) and was also free of smaller, more hydrophilic peptides (based on RP-HPLC retention time).

Several other scaleable fractionation methods (precipitation, ultrafiltration and chromatographic techniques) were tried to achieve a further fractionation of IEC2HIC3, but none of them resulted in an efficient separation of the peptides (results not shown).

Identification of the βLg peptides

Figure 3 shows the semi-preparative RP-HPLC chromatogram of IEC1. The codes of the peaks refer to Table 1, which shows the ESI-MS results of the peptides present in this fraction. The identification was based on the masses determined in combination with the primary structure of βLg and the specificity of plasmin. All peaks present in IEC1 could be identified, except for peak 6 (Figure 3). The latter peptide may result from non-specific hydrolysis (Table 1) but no identification purely based on the mass determined can be given. It appeared that the peptides present in IEC1 were all small peptides (< 2400 Da) and none of the identified peptides contained a cysteine residue.

Figure 4 shows the semi-preparative RP-HPLC chromatogram of IEC2HIC3. From the figure it is seen that fraction IEC2HIC3 contains many different peaks, of which 50 have been collected by semi-preparative RP-HPLC and the masses of the peptide components present have been determined. The masses (ESI-MS) of the samples allowed the identification of only a few peptides (i.e. some of the peptide-material present in peaks 18-22/26/29/36, see below). To identify the other peptides, the disulphide bonds present had to be split using reduction with DTT. As an example, Figure 5 shows the MALDI-TOF MS spectra obtained for the material from peak 4 of Figure 4 before and after reduction. The spectrum of the non-reduced sample contained one main peak (protonated molecule 7914.9 Da). The spectrum of the reduced sample contained two main peaks (protonated molecules 2475.9 Da and 5439.3 Da) besides the small peak with the same mass as the non-reduced peptide. These two peaks could be identified by using the masses determined, the primary structure of βLg and the specificity of plasmin (i.e. βLg[f 142-162], calculated mass 2478.9 Da, and βLgB[f 92-138], calculated mass 5442.2 Da). The non-reduced peptide appeared to be these two fragments linked together by an

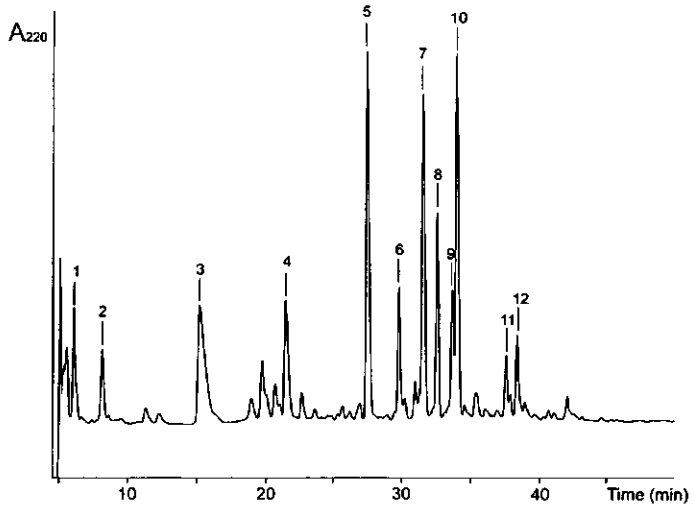

Figure 3. Semi-preparative RP-HPLC chromatogram of IEC1; RP-HPLC gradient RP1. Code of the peaks refers to Table 1.

Table 1. ESI-MS results for the peptide components of fraction IEC1, as separated by RP-HPLC (Figure 3).

RP-HPLC peak	Measured value (Da)	Peptide sequence (from both genetic variant A and B)	Calculated value (Da)
1	330.3	[f 139-141]	330.2
2	572.3	[f 71-75]	572.3
3	916.0	[f 84-91]	915.4
4	672.3	[f 9-14]	672.3
5	932.6	[f 1-8]	932.5
6	652.5	*	
7	903.1	[f 76-83]	902.5
8	1065.2	[f 92-100]	1064.5
9	1585.5	[f 70-83]	1585.0
10	1457.3	[f 71-83]	1456.9
11	1800.7	[f 76-91]	1801.1
12	2355.4	[f 71-91]	2355.8

* When specificity of plasmin was taken into account, no fragment was found for this measured mass. When the specificity of the enzyme was ignored, several peptides could be assigned (with an accuracy of at least 2 Da): AB[f 144-148], AB[f 42-46], AB[f 97-101], AB[f 106-111] and AB[f 133-137], with calculated masses of 652.8 Da, 651.7 Da, 653.7 Da, 653.7 Da and 650.7 Da, respectively.

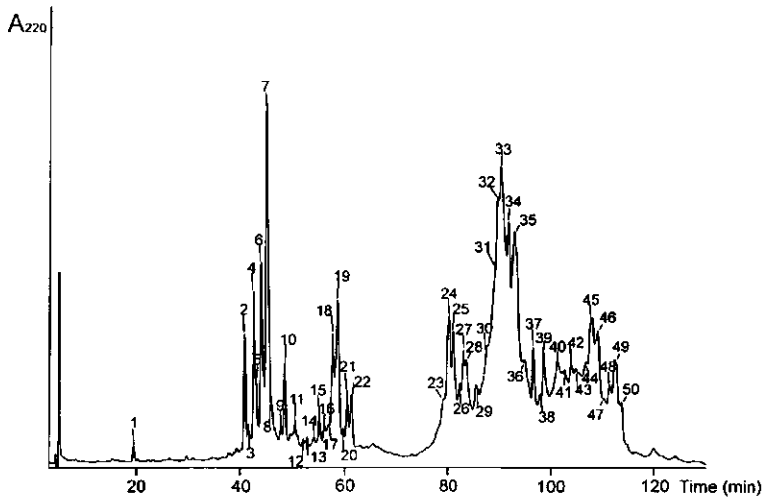

Figure 4. Semi-preparative RP-HPLC chromatogram of IEC2HIC3; RP-HPLC gradient RP2. Code of the peaks refers to Appendix I (p. 94) and Table 2.

Table 2. A summary of MS results for the peptide components of fraction IEC2HIC3, as separated by RP-HPLC (Figure 4); complete results are shown in Appendix I (p. 94).

HPLC peaks	HPLC retention time (min)	Sequences identified (mostly from both genetic variant A and B)
2-7	40.4-46.1	[f 139/142-162] -S-S- [f 84/92/101-135/138, SS][a]
10-12/15	48.2-52.1/54.9-55.5	[f 142-162] -S-S- [f 71/76/92/101/102-138/141, SS][a]
18-22	57.2-61.5	[f 70/71/76/84/92-162, 2SS][b]
24-25	79.5-81.8	[f 15-70/83] -S-S- [f 92/101-138, SS]
26	82.0-82.6	[f 15-60][c]
27-29	82.8-85.7	[f 142/149-162] -S-S- [f 15-69/70]
		[f 101-141, SS] -S-S- [f 101-141, SS]
		[f 101-141, SS] -S-S- [f 92-138, SS]
		[f 71-162, 2SS][b]
31-34	87.9-92.3	[f 84/92/101-138/141, SS] -S-S- [f 9-69/70/83/91][d]
		[f 76-135, SS] -S-S- [f 84-138, SS]
35-36	92.3-95.4	[f 9-70] -S-S- [f 142-162]
		[f 9-60][c]
41	101.9-102.9	[f 15-70] -S-S- [f 15-70]
44-46	106.3-109.6	[f 9/15-69/70] -S-S- [f 9-69/70/77]
49	111.9-113.1	[f 9-70] -S-S- [f 9-70]

[a] A variant elutes later than B variant.

[b] sequence contains only intramolecular disulphide bond

[c] sequence without Cys-residue.

[d] sequences including [f 9-69/70] elute later than those including [f 9-83/91].

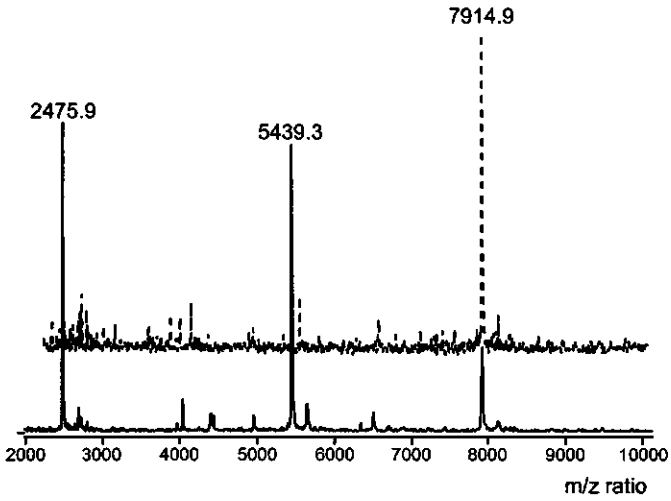

Figure 5. MALDI-TOF MS spectra of the peptide component present in peak 4 of the RP-HPLC chromatogram shown in Figure 4; dashed line for non-reduced sample and solid line for reduced sample.

intermolecular disulphide bond (with fragment βLgB[f 92-138] also having an intramolecular disulphide bond), resulting in a calculated mass of 7916.1 Da (protonated form). All other peaks collected from IEC2HIC3 (Figure 4) were analysed in the same way and most of the peaks could be identified. The MS results and the identification of the peptide components present in IEC2HIC3 (Figure 4) are shown in Appendix I (p. 96) and in Table 2, the latter being a summary of the results presented in Appendix I (p. 96). The results show that many peptides are fragments linked by newly formed disulphide bonds; only a few of the peptides are linked by the original disulphide bonds present in βLg between residues 66 and 160 (i.e. βLg[f 9/15-69/70] -S-S- βLg[f 142/149-162].

Functional properties

Figure 6 shows the results of the foam screening test (performed at 0.005% w/v). The DH4 hydrolysate formed more foam than βLg did (as shown before at 0.01% w/v, Chapter 6). Fraction IEC2HIC3, containing the larger peptides with many hydrophobic groups (see above), had the best foam-forming properties. The fractions IEC2HIC1 and IEC2HIC2, containing mainly intact βLg and some smaller, more hydrophilic peptides (Figure 2c), had even worse foam-forming properties than βLg. The foam formed by IEC1 was extremely unstable. The foam-stabilising properties of all other fractions was good; no coalescence was observed during the measurements (60 min). Under reducing conditions, the foam-forming properties of βLg increased, whereas those of IEC2HIC3 decreased; reduction had no

measurable influence on the foam-stabilising properties of these samples (no further results shown).

Table 3 shows the results of the emulsion screening test. At the low protein concentration used (0.05% w/v) only the emulsions formed by the DH4 hydrolysate and the fractions IEC2, IEC2HIC2 and IEC2HIC3 had a uniform particle-size distribution (of which the DH4 hydrolysate, IEC2 and IEC2HIC3 formed the smallest droplets). The other fractions (βLg, IEC1 and IEC2HIC1) formed emulsions containing a double peak in the particle-size distribution (indicating the presence of smaller and larger droplets at the same time; Chapter 6). At this low concentration, the emulsions formed by the latter fractions were rather unstable and showed creaming after 1h. The other fractions formed stable emulsions (according to the turbidity measurements), showing only a minor amount of creaming after 24h (IEC2HIC3 was most stable against creaming). At higher peptide concentration (0.2%) the emulsions formed with the DH4 hydrolysate and with the fractions IEC2 and IEC2HIC3 possessed similar stability to that of intact βLg (no further results shown).

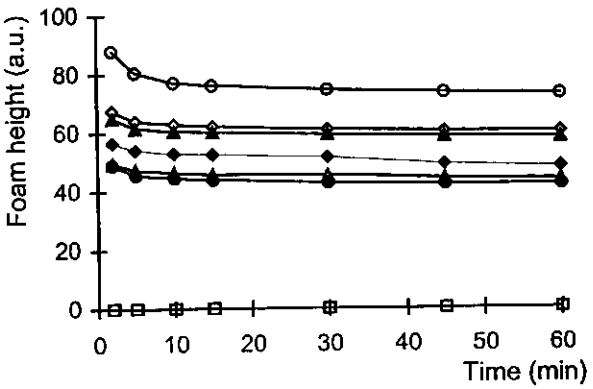

Figure 6. Foam height as produced with the various βLg fractions (Figure 1, 2) at pH 6.7, as a function of time after whipping (means of duplicate measurements), intact βLg (◆), DH4 hydrolysate (◇), IEC1 (□), IEC2 (▲), IEC2HIC1 (△), IEC2HIC2 (●), IEC2HIC3.

Discussion

SH/SS exchange

Although a large number of peptides was present in IEC1 and IEC2HIC3, RP-HPLC in combination with MS enabled almost complete identification of the peptides present. From the results in Appendix I (p. 94) it appeared that approximately 10% of the peptides present in IEC2HIC3 (proportion estimated from RP-HPLC peak area) could be identified without the presence of reduction agent, owing to the fact

Table 3. Screening test results of emulsions made at pH 6.7 with βLg hydrolysate fractions[a].

Sample	d_{32}[b] (1st/2nd [μm] peak)[c]	Stability[d]
Concentration	*0.05%*	
βLg	2.8 (2/6)	±
DH4 hydrolysate	2.0	+
IEC1	6.7 (3/14)	--
IEC2	2.1	+
IEC2HIC1	2.9 (2/10)	-
IEC2HIC2	2.4	±
IEC2HIC3	1.9	+

[a] For abbreviations used, see text.

[b] d_{32} is the average particle-size of the emulsion droplets.

[c] The measurement of the average particle size had a double peak (values of the separate peaks).

[d] +, ± and - indicate the extent of stability.

that they either did not contain a cysteine residue, i.e. βLg[f 9/15-60], or had just intramolecular disulphide bonds, e.g. βLgA[f 70/71/76/84/90-162]. Other peptides identified were combinations of 2 separate fragments of the βLg molecule linked by an intermolecular disulphide bond. One of the masses determined in peak 39 (Figure 4) indicated the presence of traces of intact βLgB; no traces of βLgA were found.

The enormous number of peptides formed during hydrolysis of βLg as shown in Table 1 and Appendix I (p. 94) can tentatively be classified as follows:

1. peptides composed of a single amino acid chain without cysteine residues (e.g. βLg[f 9/15-60]);
2. peptides composed of a single amino acid chain containing 2 or 4 cysteine residues, and having intramolecular disulphide bonds (e.g. βLgA[f 70/71/76/84/90-162]);
3. peptides composed of 2 amino acid chains, each containing 1 or 3 cysteine residues, linked by an intermolecular disulphide bond; these intermolecular disulphide bonds can either be originally present or newly formed.

Figure 7 shows a schematic picture of the peptides, mostly disulphide linked, originating from βLgA and present in IEC2HIC3 following the above-mentioned classification. Peptides comprised of 2 fragments linked by an original βLg disulphide bond (between 66-160) were identified previously in βLg/trypsin hydrolysates (Dalgalarrondo et al., 1990; Turgeon et al., 1992; Chen et al., 1993; Otte et al., 1997a). Recently, a βLg peptide having a mass of 4302 Da was assigned to the sequence βLg[f 102-124] -S-S- [f 149-162] having a newly formed disulphide bond (probably between the free thiol group, residue 121, and the cysteine residue 160 present in the [f 149-162] subsequence; Maynard et al., 1998). Part of the peptides identified in the present study might contain original βLg disulphide bonds

(between 66-160 and 106-119). However, many peptides also contain newly formed disulphide bonds, since the peptides having 1 or 3 cysteine residues appear to be extremely reactive in forming new disulphide linkages (Appendix I, p. 94). Peptides from the N-terminal half of the molecule, βLg[f 10/15-69/70], are linked to peptides of the middle part of the molecule, βLg[f 71/77/91/101/102-138/141], in many different combinations with a newly formed intermolecular disulphide bond between residues 66 and 106, 119 or 121. Apparently, a lot of reshuffling of disulphide bonds has taken place during the plasmin hydrolysis of βLg. Since the pKa of the thiol group is rather high (approximately 9.0-9.5, depending on its surroundings; Creighton, 1993), the reactivity of the thiol group is negligible at neutral pH. Therefore, it is concluded that the reshuffling took place during hydrolysis (pH 8) rather than during fractionation (pH 6 and 7). It is remarkable that fragment βLg[f 142-162] is linked to almost all other fragments formed. Apparently, this fragment is quite reactive in forming new disulphide bonds. In the native βLg, the original disulphide bond between residues 66 and 160 is on the outer surface, whereas the other disulphide bond (106-119) is buried in the interior of the native molecule (Papiz et al., 1986). The exposure of the disulphide bond might be a reason for its reactivity (Hoffmann and Van Mil, 1997). This may also hold for the reactivity of βLg[f 142-162].

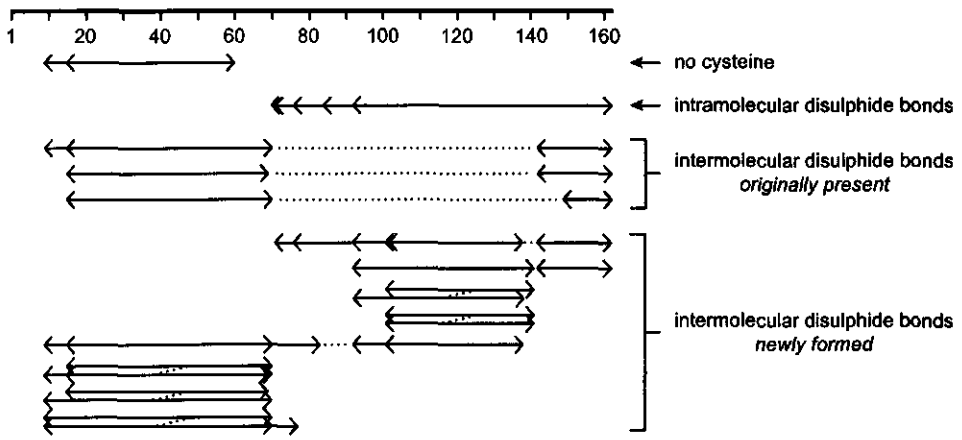

Figure 7. Schematic representation of the βLgA peptides present in IEC2HIC3; dashed line indicates intermolecular disulphide bond.

The peptides identified in this study never consisted of more than two intermolecular disulphide-linked βLg fragments. So, although SH/SS exchange does take place during βLg hydrolysis, this seems not to result in large peptide aggregates, which is in agreement with the literature (Otte et al., 1997b). Although disulphide exchange reactions are thought to play an initiating role in the thermally induced aggregation of βLg, their role is considered to be minor compared with other

factors involved such as hydrophobic and electrostatic interactions (Roefs and De Kruif; 1994; Sawyer et al., 1994). Also, Otte et al. (1997b) showed that mainly the non-covalent interactions were the interacting forces in the aggregates formed during βLg hydrolysis with proteases from *Bacillus licheniformis*.

Some peptides present in IEC2HIC3 eluted in two different regions of the RP-HPLC chromatogram (Appendix I, p. 94). For instance, peptide βLg[f 142-162] -S-S- βLgB[f 101-138, SS] appeared in peak 2/3 and in peak 10 of Figure 4 (retention times 40.4 - 41.9 min and 48.2 - 48.8 min, respectively). A similar phenomenon could be observed for the peptide βLg[f 142-162] -S-S- βLgB[f 92-138, SS] (appearing in the peaks 4/5 and 15 of Figure 4) and for the peptide βLgA[f 71-162, 2SS] (eluting in the peaks 22 and 29 of Figure 4). A possible explanation might be that different disulphide bonds are present within the peptide. For instance, in the case of the latter example different combinations between the cysteine residues present at positions 66, 119, 121 and 160 are possible, which will result in an altered surface hydrophobicity of the total peptide and consequently in a different RP-HPLC retention behaviour.

Fractionation

Fractionation of βLg DH4 hydrolysate resulted in several peptide fractions. Fraction IEC1 contained a mixture of smaller and/or hydrophilic peptides (Table 1), and fraction IEC2HIC3 contained a complex mixture of larger peptides with many hydrophobic groups and many of the peptides contained disulphide bonds (Appendix I, p. 94). Further fractionation of IEC2HIC3 by scaleable methods was not successful (results not shown). Other papers (Turgeon et al., 1992; Chen et al., 1993) described the fractionation (by ultrafiltration and by IEC) of tryptic βLg hydrolysates, in which no SH/SS reshuffling was observed although the hydrolysis had also been performed at pH 8. The peptides identified by Turgeon et al. (1992) were smaller than the peptides present in IEC2HIC3, which resulted in more peptides lacking a cysteine residue. Probably, both the presence of smaller peptides and the absence of SH/SS exchange made it possible to fractionate that tryptic hydrolysate. Chen et al. (1993) hydrolysed βLg at low temperature (5-10°C), which might have prevented reshuffling in the hydrolysate, despite the larger size of the peptides (6.7, 9.6 and 13.8 kDa) and the presence of cysteine residues in these peptides. In our previous study (Chapter 6) it was shown that the βLg hydrolysates formed by the action of trypsin and *Staphylococcus aureus* V8 protease contained less disulphide-linked peptides than the hydrolysates formed by the action of plasmin (as determined by gel-permeation chromatography under denaturing + reducing conditions). Probably, the use of a less selective enzyme than plasmin results in a βLg hydrolysate containing smaller peptides of which many lack a cysteine residue. Therefore, a large number of peptides is present that cannot take place in the SH/SS exchange. Hence, more distinct differences between several peptides will exist, which might enable the fractionation of such a hydrolysate.

Functional properties

The DH4 hydrolysate already had improved foam-and emulsion-forming properties, when compared to βLg. Fraction IEC1, containing smaller peptides (mostly < 2 kDa), had poor foam- and emulsion-forming and -stabilising properties. These results are in agreement with the concept that a minimum molecular weight of 2000 Da is essential for good interfacial and emulsifying properties (Turgeon et al., 1992). Fraction IEC2HIC3 was formed after removing mainly intact βLg with IEC2HIC1 and IEC2HIC2 from IEC2 and contained larger peptides (mostly between 7 and 14 kDa). Fraction IEC2HIC3 possessed improved foam- and emulsion-forming properties and similar foam- and emulsion-stabilising properties when compared to βLg, DH4 hydrolysate and IEC2.

Reduction of the disulphide bonds in βLg seemed to improve its foam-forming properties but in IEC2HIC3 to decrease foam-forming properties (results not shown). By reducing the disulphide bonds in βLg, the molecule becomes a more flexible structure, which apparently favours the foam-forming properties. The molecules present in IEC2HIC3 already have a more flexible structure (due to the hydrolysis), which could explain the improved functionality. The reduction of the disulphide bonds in IEC2HIC3 resulted in smaller peptides (many between 2 and 7 kDa), possessing decreased functional properties. Earlier, it was suggested that the poor interfacial properties of a βLg peptide fraction that mainly contained disulphide-linked peptides was caused by the rigid structure due to the disulphide bond (Turgeon et al., 1992). However, in our study it was found that the presence of many disulphide bonds in IEC2HIC3 was not detrimental for the functional properties of this peptide fraction.

In conclusion, it is shown that most of the peptides present in a βLg/plasmin hydrolysate are disulphide-linked βLg fragments and considerable SH/SS exchange takes place during plasmin hydrolysis of βLg. In contrast to the earlier reports on the negative effect of the presence of disulphide bonds on the interfacial properties (Turgeon et al., 1992), the results presented in this Chapter showed good functional properties of a peptide fraction containing many disulphide bonds.

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APPENDIX I-1

ESI-MS and MALDI-TOF MS results for the peptide components of fraction IEC2HIC3, as separated by RP-HPLC (Figure 4)

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
1	x			x		
2	2477.8	AB[f 142-162]	2477.9	6867.1	AB[f 142-162] -S-S- B[f 101-138, SS]	6867.9
	4392.9	B[f 101-138]	4394.0			
3	2478.4	AB[f 142-162]	2477.9	6867.3[b]	AB[f 142-162] -S-S- B[f 101-138, SS]	6867.9
	2789.9	AB[f 139-162]	2791.3	7179.3	AB[f 139-162] -S-S- B[f 101-138, SS]	7180.3
	4395.5	B[f 101-138]	4394.0	7524.0	A[f (142-162] -S-S- B[f 92-135, SS]	7524.7
	5050.6	B[f 92-135]	5050.8	7957.7[c]		
4	2477.8	AB[f 142-162]	2477.9	7914.2	AB[f 142-162] -S-S- B[f 92-138, SS]	7915.1
	5442.6	B[f 92-138]	5441.2			
5	2478.2	AB[f 142-162]	2477.9	6899.3	AB[f 142-162] -S-S- A[f 101-138, SS]	6895.8
	2789.9	AB[f 139-162]	2790.3	7914.1	AB[f 142-162] -S-S- B[f 92-138, SS]	7915.1
	4423.9	A[f 101-138]	4422.1	8226.0	AB[f 139-162] -S-S- B[f 92-138, SS]	8227.5
	5443.4	B[f 92-138]	5441.2	8811.9	AB[f 142-162] -S-S- B[f 84-138, SS]	8813.1
	6342.7	B[f 84-138]	6339.2			
6	2481.7	AB[f 142-162]	2477.9	6895.1	AB[f 142-162] -S-S- A[f 101-138, SS]	6895.8
	4419.6	A[f 101-138]	4422.1			
	6891.4[d]	A[f 9-70]	6890.9		A[f 9-70]SH[d,e]	6891.9[e]
7	2475.6	AB[f 142-162]	2477.9	7942.3	AB[f 142-162] -S-S- A[f 92-138, SS]	7943.2
	5465.8	A[f 92-138]	5469.3			
	7940.4[d]					
8	2478.3[b]	AB[f 142-162]	2477.9	7942.8[b]		
	2703.1[c]			8254.2[c]		
	5442.2	B[f 92-138][f]	5441.2			
9	2476.8[b]	AB[f 142-162]	2477.9	7207.0[c]		
				8578.1[c]		
				10379.9[c]		
10	2477.7	AB[f 142-162]	2477.9	6767.6	AB[f 142-162] -S-S- A[f 102-138, SS]	6767.8
	4295.6	A[f 102-138]	4293.9	8254.6	AB[f 142-162] -S-S- A[f 92-141, SS]	8255.6
	5784.4	A[f 92-141]	5781.7	9151.8[c]		
	6771.3[d]			10251.8[c]		
11	2479.6	AB[f 142-162]	2477.9	6865.5	AB[f 142-162] -S-S- B[f 101-138, SS]	6867.9
	4392.7	B[f 101-138]	4394.0	9726.0	AB[f 142-162] -S-S- A[f 76-138, SS]	9725.3
	4421.7	A[f 101-138][f]	4422.1	10281.1	AB[f 142-162] -S-S- A[f 71-138, SS][h]	10280.0
	5444.8	B[f 92-138][f]	5441.2	15529.8[c]		
	5466.3	A[f 92-138][f]	5469.3			
	7249.5	A[f 76-138]	7252.4			
	7307.3	B[f 78-141][f]	7307.5			
	7804.4[g]	A[f 1-70][g]	7806.0			
		A[f 71-138][g]	7807.1			
	9726.1[d]					
	10278.3[d]	A[f 9-100]	10275.9		A[f 9-100]SH[d,e]	10275.9[e]
12	x			7895.8[c]		
				10280.6[b]	AB[f 142-162] -S-S- A[f 71-138, SS][h]	10280.0
				10720.9[c]		
13	x			10037.8[c]		
				10592.3[c]		
14	x			6021.1[c]		
				8829.7[c]		
15	2478.0	AB[f 142-162]	2477.9	7923.3	AB[f 142-162] -S-S- B[f 92-138, SS]	7915.1
	4394.3	B[f 101-138][f]	4394.0	8129.7	B[f 78-148, SS]SH[e]	8124.5[e]
	4421.3	A[f 101-138][f]	4422.1	8856.1[c]		
	5447.2	B[f 92-138]	5441.2			
	5468.9	A[f 92-138][f]	5469.3			
	7925.4[d]					

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
16	2478.6	AB[f 142-162][b,f]	2477.9	8827.6[c]		
	4395.5	B[f 101-138][f]	4394.0	9381.9[b,c]		
	4426.2	A[f 101-138][f]	4422.1			
	8834.7[d]					
	9396.5[d]					
17	x			7773.0	B[f 71-138, SS]SH[e]	7777.0[e]
				8560.7	B[f 61-135, SS]SH[e]	8560.9
				9168.1	B[f 9-91]SH[e]	9170.6[e]
				9382.2[b,c]		
18	8209.3	B[f 92-162]	8213.5	8208.6	B[f 92-162, 2SS]	8209.5
	9108.4	B[f 84-162]	9111.5	9106.2	B[f 84-162, 2SS]	9107.5
19	8239.2	A[f 92-162]	8241.57	8236.2	A[f 92-162, 2SS]	8237.6
	9136.9	A[f 84-162]	9139.6	9134.5	A[f 84-162, 2SS]	9135.6
20	7731.3			7736.1[c]		
	8236.1[b]			8237.2[b]		
	10673.7	B[f 70-162]	10679.5	10674.2	B[f 70-162, 2SS]	10675.5
21	9996.0	B[f 76-162]	9996.6	9990.4	B[f 76-162, 2SS]	9992.6
	10551.2	B[f 71-162]	10551.3	10545.3	B[f 71-162, 2SS]	10547.3
	10711.7	A[f 70-162]	10707.5	10702.3	A[f 70-162, 2SS]	10703.5
22	10019.3	A[f 76-162]	10024.7	10019.3	A[f 76-162, 2SS]	10020.7
	10574.5	A[f 71-162]	10579.4	10573.9	A[f 71-162, 2SS]	10575.4
23	x			x		
24	4394.3	B[f 101-138]	4394.0	7616.9[i]		
	4423.6	A[f 101-138]	4422.1	10570.3	B[f 15-70] -S-S- B[f 101-138, SS]	10568.1
	5441.4	B[f 92-138]	5441.2	10594.6	B[f 15-70] -S-S- A[f 101-138, SS]	10596.3
	5467.7	A[f 92-138]	5469.3	11615.6	B[f 15-70] -S-S- B[f 92-138, SS]	11615.3
	6178.1	B[f 15-70]	6178.1	11642.7	B[f 15-70] -S-S- A[f 92-138, SS]	11643.3
	6232.2	A[f 15-70][b,f]	6236.1	12009.1	B[f 15-83][h] -S-S- B[f 101-138, SS]	12006.8
	7619.5[g]	B[f 1-69][g]	7619.8	12035.8	B[f 15-83][h] -S-S- A[f 101-138, SS]	12034.9
		B[f 9-77][g]	7616.8	13056.6	B[f 15-83][h] -S-S- B[f 92-138, SS]	13054.0
		B[f 15-83][g]	7617.8	13084.8	B[f 15-83][h] -S-S- A[f 92-138, SS]	13082.1
25	2477.2	AB[f 142-162][g]	2477.9	10653.0	A[f 15-70] -S-S- A[f 101-138, SS]	10654.2
	3205.4[c]			11700.7	A[f 15-70] -S-S- A[f 92-138, SS]	11701.4
	4419.4	A[f 101-138]	4422.1	12095.8	A[f 15-83][h] -S-S- A[f 101-138, SS]	12092.0
	5466.8	A[f 92-138]	5469.3	13140.6	A[f 15-83][h] -S-S- A[f 92-138, SS]	13141.2
	6240.8	A[f 15-70]	6236.1			
	7675.4[g]	A[f 1-69][g]	7677.9			
		A[f 9-77][g]	7674.8			
		A[f 15-83][g]	7675.9			
26	2477.8	AB[f 142-162][b,f]	2477.9	5002.7	AB[f 15-60]	5003.8
	4395.7	B[f 101-138][f]	4394.0			
	4426.5	A[f 101-138][b,f]	4422.1			
	5007.3	AB[f 15-60]	5003.8			
	5447.1	B[f 92-138][f]	5441.2			
	5471.2	A[f 92-138][b,f]	5469.3			
	6053.6	B[f 15-69][f]	6049.9			
	6113.8	A[f 15-69][f]	6107.9			
	6184.0	B[f 15-70][b,f]	6189.3			
	6239.8	A[f 15-70][b,f]	6236.1			
27	2475.4	AB[f 142-162]	2477.9	4634.5[i]		
	4632.5	A[f 101-141]	4635.4	8652.7	AB[f 142-162] -S-S- B[f 15-70]	8654.0
	5469.2	A[f 92-138]	5469.3	8710.9[b]	AB[f 142-162] -S-S- A[f 15-70]	8712.0
	6177.1	B[f 15-70]	6178.1	9266.8	A[f 101-141, SS]-S-S-A[f 101-141, SS]	9264.7
	6236.4	A[f 15-70]	6236.1	10092.3	A[f 101-141, SS] -S-S- A[f 92-138, SS]	10098.7
28	1661.3	AB[f 149-162]	1658.9	7896.2	AB[f 149-162] -S-S- A[f 15-70]	7893.0
	2478.4	AB[f 142-162]	2477.9	8711.4[b]	AB[f 142-162] -S-S- A[f 15-70]	8712.0
	4394.7	B[f 101-138][b,f]	4394.0			
	6239.8	A[f 15-70]	6236.1			

APPENDIX I-3

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
29	2478.0	AB[f 142-162]	2477.9	8586.5	AB[f 142-162] -S-S- A[f 15-69]	8583.8
	4394.6	B[f 101-138][b,f]	4394.0	10578.4	A[f 71-162, 2SS]	10575.4
	4421.2	A[f 101-138][b,f]	4422.1	10735.8[c]		
	5442.0	B[f 92-138][b,f]	5441.2			
	5470.1	A[f 92-138][b,f]	5469.3			
	6110.4	A[f 15-69]	6107.9			
30	2477.3	AB[f 142-162][b]	2477.9	10054.3[c]		
	4394.0	B[f 101-138][b,f]	4394.0	10604.7[c]		
	4424.7	A[f 101-138][b,f]	4422.1			
	5441.3	B[f 92-138][b,f]	5441.2			
	5469.4	A[f 92-138][b,f]	5469.3			
31	2478.4	AB[f 142-162][f]	2477.9	8271.0[i]		
	4394.6	B[f 101-138]	4394.0	13168.4	B[f 84-138, SS] -S-S- B[f 76-135,, SS]	13167.1
	4422.4	A[f 101-138]	4422.1	13196.9	A[f 84-138, SS] -S-S- B[f 76-135, SS]	13195.2
	5441.7	B[f 92-138]	5441.2	13560.7	B[f 9-91] -S-S- B[f 101-138, SS]	13560.6
	5470.2	A[f 92-138]	5469.3	13589.2	B[f 9-91] -S-S- A[f 101-138, SS]	13588.7
	5757.9	B[f 92-141]	5757.9	14054.3	B[f 9-83] -S-S- A[f 92-141, SS]	14050.3
	5772.7	A[f 92-141]	5781.7	14606.7	B[f 9-83] -S-S- B[f 84-138, SS]	14607.9
	6340.4	B[f 84-138]	6339.2		B[f 9-91] -S-S- B[f 92-138, SS]	14607.8
	6367.5	A[f 84-138]	6367.3	14636.8[g]	B[f 9-83] -S-S- A[f 84-138, SS]	14634.9
	6655.3	B[f 84-141]	6651.6		B[f 9-91] -S-S- A[f 92-138, SS]	14635.9
	6834.4	B[f 76-135]	6833.9	14921.9[g]	B[f 9-83] -S-S- B[f 84-141, SS]	14920.3
	8275.3	B[f 9-83]	8272.6		B[f 9-91] -S-S- B[f 92-141, SS]	14920.3
	9172.0	B[f 9-91]	9170.6			
32	2477.7	AB[f 142-162][f]	2477.9	8270.9[i]		
	4396.4	B[f 101-138]	4394.0	12667.3	B[f 9-83] -S-S- B[f 101-138, SS]	12662.6
	4422.4	A[f 101-138]	4422.1	12690.6	B[f 9-83] -S-S- A[f 101-138, SS]	12690.7
	5444.5	B[f 92-138]	5441.2	13711.1	B[f 9-83] -S-S- B[f 92-138, SS]	13709.9
	5470.7	A[f 92-138]	5469.3	13736.0	B[f 9-83] -S-S- A[f 92-138, SS]	13737.9
	8274.1	B[f 9-83]	8272.6			
33	2478.6	AB[f 142-162][b,f]	2477.9	11227.3	B[f 9-70][h] -S-S- B[f 101-138, SS]	11222.8
	2791.5	AB[f 139-162][b,f]	2790.3	11253.9	B[f 9-70][h] -S-S- A[f 101-138, SS]	11250.9
	4394.5	B[f 101-138]	4394.0	11282.7	A[f 9-70] -S-S- B[f 101-138, SS]	11280.9
	4422.1	A[f 101-138]	4422.1	11312.3	A[f 9-70] -S-S- A[f 101-138, SS]	11308.9
	5441.9	B[f 92-138]	5441.2	12272.7	B[f 9-70][h] -S-S- B[f 92-138, SS]	12270.1
	5468.3	B[f 92-138]	5469.3	12299.9	B[f 9-70][h] -S-S- A[f 92-138, SS]	12298.1
	6832.4[f]	B[f 9-70][g]	6832.8	12326.6	A[f 9-70] -S-S- B[f 92-138, SS]	12328.1
		B[f 76-135][g]	6833.9	12359.9	A[f 9-70] -S-S- A[f 92-138, SS]	12356.1
	6892.6	A[f 9-70]	6890.9	12670.0	B[f 9-83] -S-S- B[f 101-138, SS]	12662.6
	8272.8	B[f 9-83]	8272.6	12724.5	A[f 9-83] -S-S- B[f 101-138, SS]	12720.7
	8328.5	A[f 9-83]	8330.7	12749.1	A[f 9-83] -S-S- A[f 101-138, SS]	12748.8
				13797.9	A[f 9-83] -S-S- A[f 92-138, SS]	13796.0
34	2477.7	AB[f 142-162][b,f]	2477.9	11125.9	B[f 9-69] -S-S- A[f 101-138, SS]	11122.8
	2789.5	AB[f 139-162][b,f]	2790.3	11313.5[b]	A[f 9-70] -S-S- A[f 101-138, SS]	11308.9
	4394.5	B[f 101-138][b,f]	4394.0	12171.2	B[f 9-69] -S-S- A[f 92-138, SS]	12170.0
	4420.6	A[f 101-138]	4422.1	14591.0[c]		
	5442.9	B[f 92-138][b,f]	5441.2	14819.7[c]		
	5469.7	A[f 92-138]	5469.3	14903.2[c]		
	6706.5	B[f 9-69]	6704.7	19840.4[c]		
	6834.0[g]	B[f 9-70][b,f,g]	6832.8			
		B[f 76-135][g]	6833.9			
	6893.3	A[f 9-70][b,f]	6890.9			
	7294.2[c]					
	8274.5	B[f 9-83][b,f]	8272.6			
	9171.2	B[f 9-91][b,f]	9170.6			
35	2476.7	AB[f 142-162]	2477.9	9310.6	AB[f 142-162] -S-S- B[f 9-70]	9309.8
	6834.1[g]	B[f 9-70][g]	6832.8	9368.4	AB[f 142-162] -S-S- A[f 9-70]	9366.8
		B[f 76-135][g]	6833.9	10747.6[c]		
	6889.8	A[f 9-70]	6890.9	20751.5[c]		

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
36	2476.3	AB[f 142-162][b]	2477.9	5657.4	AB[f 9-60]	5658.5
	2489.3	AB[f 70-91][f]	2484.0	9182.9	B[f 1-83]SH[e]	9187.8[e]
	4393.9	B[f 101-138][f]	4394.0	9237.5[c]		
	5656.7	AB[f 9-60]	5658.5	10281.1[c]		
37	2477.7	AB[f 142-162][b,f]	2477.9	5288.2[c]		
	5290.9[c]			5497.7[c]		
	5497.1[c]					
38	x			6203.2[c]		
				18343.0[c]		
39	2479.3	AB[f 142-162][f]	2477.9	5529.5[i]		
	5531.4	B[f 101-148][f]	5525.5	17358.5[c]		
	6179.2	B[f 15-70][f]	6178.1	18276.9	B[f 1-162, 2SS]SH[e]	18277.3[e]
	6237.5	A[f 15-70][f]	6236.1			
40	2480.5	AB[f 142-162][f]	2477.9	8722.8[c]		
	6180.1	B[f 15-70][b,f]	6178.1			
	6238.1	A[f 15-70][b,f]	6236.1			
	6893.4	A[f 9-70][f]	6890.9			
41	2479.3	AB[f 142-162][f]	2477.9	12410.2	A[f 15-70] -S-S- B[f 15-70]	12412.2
	6180.4	B[f 15-70]	6178.1	12468.7	A[f 15-70] -S-S- A[f 15-70]	12470.2
	6238.5	A[f 15-70]	6239.1			
42	4397.8	B[f 101-138][f]	4394.0	12243.7[c]		
	4421.4	A[f 101-138][f]	4422.1	12382.1[c]		
	5444.1	B[f 92-138][f]	5441.2			
	5470.9	A[f 92-138][f]	5469.3			
	6107.6	A[f 15-69][f]	6107.9			
	6178.7	B[f 15-70][f]	6178.1			
	6235.1	A[f 15-70][f]	6236.1			
	6836.58	B[f 9-70][f,g]	6832.8			
		B[f 76-135][f,g]	6833.9			
	6889.1	A[f 9-70][f]	6890.9			
43	x			x		
44	6183.0	B[f 15-70][b,f]	6178.1	14448.2	B[f 9-70] -S-S- B[f 9-77][h]	14447.6
	6234.1	A[f 15-70][b,f]	6236.1	14505.0[h]	B[f 9-70] -S-S- A[f 9-77][h]	14505.7
	6834.3g	B[f 9-70]g	6832.8		A[f 9-70] -S-S- B[f 9-77][h]	14505.7
		B[f 76-135]g	6833.9	14526.8[c]		
	6890.5	A[f 9-70]	6890.9	14562.9	A[f 9-70] -S-S- A[f 9-77][h]	14563.7
	7619.7g	B[f 1-69]g	7619.8			
		B[f 9-77]g	7616.8			
		B[f 15-83]g	7617.9			
	7675.0g	A[f 1-69]g	7677.9			
		A[f 9-77]g	7674.8			
		A[f 15-83]g	7675.9			
45	6177.4	B[f 15-70]	6178.1	13065.4g	B[f 15-70] -S-S- A[f 9-70]	13066.9
	6236.1	A[f 15-70]	6236.1		A[f 15-70] -S-S- B[f 9-70]	13066.9
	6832.6g	B[f 9-70]g	6832.8	13123.2	A[f 15-70] -S-S- A[f 9-70]	13125.0
		B[76-135]g	6833.9			
	6891.2	A[f 9-70]	6890.9			
46	6110.9	A[f 15-69]	6107.9	12938.0	A[f 15-69] -S-S- B[f 9-70][h]	12938.7
	6237.7	A[f 15-70]	6236.1	12996.1g	A[f 15-69] -S-S- A[f 9-70]	12996.8
	6766.5	A[f 9-69]	6762.7		A[f 15-70] -S-S- A[f 9-69]	12996.8
	6838.9g	B[f 9-70]g	6832.8			
		B[f 76-135]g	6833.9			
	6893.4	A[f 9-70]	6891.9			
47	x			9286.3[c]		
				11687.2[c]		
				12640.4[c]		
				12778.2[c]		
48	x			x		

APPENDIX I-5

HPLC peak	measured mass[a] reduced sample	possible fragment reduced sample	theoretical mass	measured mass[a] non-reduced sample	possible fragment non-reduced sample	theoretical mass
49	6834.4[g]	B[f 9-70][g]	6832.8	6831.7[i]		
		B[f 76-135][g]	6833.9	13720.3	A[f 9-70] -S-S- B[f 9-70]	13721.7
	6890.3	A[f 9-70]	6891.9	13778.5	A[f 9-70] -S-S- A[f 9-70]	13779.7
50	x			13650.2[c]		

x bad spectrum, no masses found
a reduced samples determined by MALDI-TOF MS;
non-reduced samples determined by MALDI-TOF MS and/or ESI MS;
both ESI-MS and MALDI-TOF MS measured masses are of the non-protonized form
b traces from former or following peak
c could not be identified
d probably mass of non-reduced peptide
e sequence contains free SH
f peptide not used to recombine non-reduced peptide
g similar mass found for more peptides
h one sequence is given; other theoretical possibilities[g]
i probably mass of reduced peptide
SS sequence contains intramolecular disulphide bond
SH sequence contains free thiol group
-S-S- two sequences linked together by intermolecular disulphide bond

Chapter 8

General discussion

Introduction

In this thesis an investigation of the formation and stabilisation of foam and emulsions (denoted as functionality) by peptides derived from β-casein (βCN) and β-lactoglobulin (βLg) is described.

βCN and βLg are both well-described, structurally different milk proteins. βCN is a flexible, random-coil structured protein without cysteine residues with a highly charged N-terminal part and a less charged, hydrophobic C-terminal part. At temperatures above 4°C and above a critical concentration, βCN forms micelles. βLg is a compact, globular protein with two disulphide bonds and a free thiol group; the hydrophobic groups are mainly buried in the interior of the protein. The difference in structural properties between these proteins is reported as a reason for their differences in functionality (Mulvihill and Fox, 1989). Although many papers on the formation and stabilisation of foam and emulsions with milk protein hydrolysates have been published (Chapter 1), it is difficult to deduce structure-function relationships. This is due to the fact that different methods were applied to investigate the functional properties and because of the use of undefined mixtures of proteins and peptides in those studies. Nevertheless, several aspects are reported to be of importance for the functionality of peptides (Chapter 1):

- the enzyme specificity determines the peptides being formed and is therefore of importance for the functionality;
- a minimal molecular size of the peptides is necessary for good functionality;
- a clustering of hydrophobic and charged areas in the peptides is mentioned to be a characteristic for good functionality;
- the presence of minor amounts of other peptides may influence the functionality of the main peptides present;
- the influence of hydrolysis on functionality is reported to be larger for whey proteins than for caseins;

Further, for a number of synthetic peptides the secondary structure seemed to be important for the functional properties (Sheehan et al., 1998).

The aim of the study described in this thesis was to elucidate structure-function relationships of peptides from βLg and βCN in regard to their foam- and emulsion-forming and -stabilising properties. Therefore, the proteins were modified by enzymatic hydrolysis, the reaction products were fractionated and characterised, and the functional properties of the peptides and peptide mixtures were determined.

This approach enabled us to discuss structure-function relationships of the peptides at a molecular level. In this Chapter the main findings obtained in the study are briefly discussed.

Influence of enzyme used on functionality of peptides formed

The influence of enzyme specificity and selectivity on the functionality of the hydrolysates formed was investigated for βLg. The enzymes used were plasmin, trypsin and *Staphylococcus aureus* V8 protease (*S.aur.*V8). Plasmin and trypsin have the same specificity but the former is more selective (preferentially attacks Lys-X instead of Arg-X) than the latter (Bastian and Brown, 1996). *S.aur.*V8 cleaves Glu-X and Asp-X bonds. At pH 4 only Glu-X bonds are hydrolysed, whereas at around pH 8 both Glu and Asp residues will be attacked (Drapeau, 1977).

Differences in functionality of total hydrolysates were observed between the several βLg hydrolysates formed by the action of trypsin, plasmin and *S.aur.*V8 (Chapter 6). In general, the foam- and emulsion-forming properties of the hydrolysates were similar or superior to those of βLg. It has been hypothesised that improved interfacial properties of (some) βLg peptides are due to the exposure of hydrophobic groups (Turgeon et al., 1992). This might result in a low surface tension and consequently in a smaller Laplace pressure, which facilitates the break-up of the particles, leading to the improved foam- and emulsion-forming properties found for the βLg hydrolysates compared to the intact protein (Chapters 6 and 7).

At very low concentration the βLg/trypsin and βLg/*S.aur.*V8 hydrolysates and the intact protein showed a bimodal particle-size distribution, but the βLg/plasmin hydrolysates had a monomodal particle-size distribution (Chapter 6). A double peak in the particle-size distribution was also described by Agboola and Dalgleish (1996) for emulsions formed by βLg/trypsin hydrolysates. These authors reported that the peptide films were ineffective in preventing coalescence and flocculation of the oil droplets already during emulsion formation, which would explain the bimodal shapes of the size distribution. However, their experimental set-up could not give information on the coalescence during emulsion-formation. Furthermore, our study showed (Chapter 6) that the double peak in the particle-size distribution was not only present in the emulsions formed by hydrolysates, but also in those formed by βLg. In general, small molecules are more capable of preventing coalescence during emulsion formation (Walstra, 1996). Consequently, the double peak cannot or can only partly be explained by the coalescence during emulsion formation.

The emulsions having a bimodal particle-size distribution creamed more rapidly than those having a monomodal particle-size distribution. This is most probably caused by the larger droplets that are present in the former emulsions. At higher concentrations, when emulsions with monomodal particle-size distributions were formed, the βLg/*S.aur.*V8 hydrolysates had poor emulsion-stabilising properties. This is probably due to the smaller apparent molecular sizes of the peptides present in

these hydrolysates, compared to those present in the other hydrolysates (Chapter 6). Agboola and Dalgleish (1996) mentioned that the stability of the emulsions formed by βLg/trypsin hydrolysates was caused by intact protein still present in the hydrolysates. However, the results in Chapter 7 showed that after removal of residual βLg from a βLg/plasmin peptide fraction (IEC2), the stability of the emulsion formed by the resulting peptide fraction (IEC2HIC3) was still similar to that of βLg. Therefore, in IEC2 (and IEC2HIC3) the stability is caused by the peptides. Probably, their rather high molecular size prevents the peptides from desorbing from the interface and the peptides present in this βLg/plasmin hydrolysate fraction apparently provide sufficient steric repulsion for producing stable systems.

Besides differences in functional properties, the βLg hydrolysates formed by trypsin, plasmin and *S.aur.*V8 also showed different aggregation behaviour (Chapter 6). The peptides present in the hydrolysates were either not linked (most evident for hydrolysates formed by *S.aur.*V8), linked by hydrophobic interactions (hydrolysates formed by trypsin and *S.aur.*V8) or linked by disulphide bonds (most evident for the plasmin hydrolysates). This aggregation behaviour will determine the peptide composition present in the foams and emulsions and thereby possibly also the functionality. Although these results give additional information about the composition of the hydrolysates, it is impossible to deduce a structure-function relationship of a complex peptide mixture as present in the total βLg hydrolysates.

Importance of primary structure

Fractionation of hydrolysates

Figure 1 shows the distribution of charged residues in βCN and βLg as well as the hydrophobicity map of these proteins. It can be seen that βCN has a distinctive highly charged N-terminal part and a C-terminal part containing many hydrophobic side chains and only a few charged groups. In addition, with the lack of a compact structure in βCN, the hydrophobic groups are exposed. It was to be expected that some particular βCN peptides differing in charge and hydrophobicity could be produced by enzymatic hydrolysis. Indeed, as shown in Chapters 2 and 3, such peptides were formed by the action of plasmin and could be easily fractionated by common preparative methods.

In βLg, a globular protein, both the charged and hydrophobic groups are more evenly distributed (Figure 1). Consequently, the βLg peptides formed will be rather alike in terms of distribution of hydrophobic and charged groups, which partly explains the poor fractionation (Chapter 7). Also, the reshuffling of disulphide bonds (Chapter 7; see below) resulted in an enormous range of disulphide-linked βLg peptides, which further complicated the fractionation of the βLg peptide fraction IEC2HIC3. Turgeon et al. (1992) described the fractionation of a tryptic βLg hydrolysate. The peptides identified in that hydrolysate were smaller with more peptides lacking a cysteine residue, than most βLg peptides identified in our fraction,

IEC2HIC3 (Chapter 7). Therefore, the hydrolysate of Turgeon et al. (1992) could more easily be fractionated by chromatographic techniques. In order to obtain larger peptides, Chen et al. (1993) slowed down the βLg/trypsin hydrolysis by using a low incubation temperature (5-10°C). In our study the production of larger fragments was achieved by using plasmin instead of trypsin (Chapter 6). It was noted (Chapter 6) that it took a longer time to reach a certain degree of hydrolysis for plasmin than for trypsin. The low temperature used in the study of Chen et al. (1993) might have prevented reshuffling of the disulphide bonds in the hydrolysate, despite the rather large size of the peptides (compared to the peptides described by Turgeon et al., 1992) and the presence of cysteine residues in those peptides.

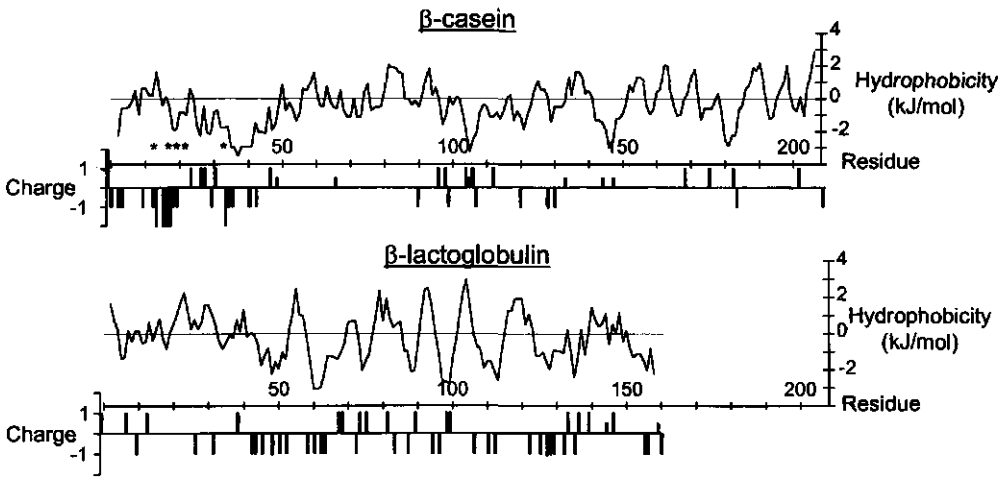

Figure 1 Schematic representation of the linear chain distribution of charged residues in βCN and βLg, as well as their hydrophobicity maps. The hydrophobicity maps are constructed according to Kyte and Doolittle (1982) with a window of 5, and without taking the various phosphoserine residues (approximate position indicated with asterisks) into account.

The βLg/plasmin hydrolysates contained more disulphide-linked peptides than the βLg/trypsin and the βLg/*S.aur.*V8 hydrolysates (Chapter 6). Combining the results from literature (Turgeon et al., 1992; Chen et al., 1993) with those presented in Chapters 6 and 7, one can predict that a careful selection of both the enzyme and the conditions of hydrolysis might result in a βLg hydrolysate in which a smaller proportion of peptides take part in the SH/SS exchange. This might facilitate the fractionation of the hydrolysate. Fractionation in the presence of a reducing agent might also facilitate the separation of the βLg peptides into several fractions. However, it should be noted that after removing the reducing agent, the cysteine-containing peptides will be rapidly linked by disulphide bridges again.

Small, hydrophilic peptides

The foam- and emulsion-stabilising properties of the small, hydrophilic peptides (from both βCN and βLg, Chapters 2 and 7, respectively) are poor, which is in line with results reported in literature (Chobert et al., 1988b; Turgeon et al., 1992). It can be hypothesised that the low surface-activity found for these peptides (shown in Chapter 2 for the hydrophilic 1-28 fragment of βCN; reported in the literature for small βLg peptides, Turgeon et al., 1992) results from a low preference for the interface due to the low hydrophobicity. Furthermore, due to their small molecular size these peptides will give less steric repulsion when adsorbed at the interface, resulting in a poor stability as well.

Amphipathic peptides

Peptides are called amphipathic when charged and hydrophobic groups are distributed in clusters. Since βLg has such a distribution of charged and hydrophobic groups (Figure 1), most βLg peptides of a certain minimal size (for instance those present in IEC2HIC3, Chapter 7) can be regarded as amphipathic. Some of the βCN peptides (i.e. βCN[f 1/29-105/107]; Chapters 2 and 3) are amphipathic in a more or less soap-like manner: with a strongly charged N-terminal end ("head") and a less charged, hydrophobic C-terminal part ("tail").

The increased foam- and emulsion-forming properties of some βLg peptides (e.g. those present in IEC2HIC3, Chapter 7) compared to those of intact βLg might be due to the more flexible structure of the peptides with exposed hydrophobic groups. This makes these molecules more amphipathic than native βLg. This will probably alter the surface activity and is apparently beneficial to the foam-forming properties. Reduction of the disulphide bonds in native βLg favoured the foam-forming properties (Chapter 7), presumably also due to the more flexible structure allowing exposure of hydrophobic groups of the molecule after reduction. It has been suggested (Turgeon et al., 1992) that the poor interfacial properties of a βLg peptide fraction, which mainly contained disulphide-linked peptides, were due to the rigid structure caused by the disulphide bonds. However, in the present study it was found that the occurrence of the many disulphide bonds in the βLg peptide fraction IEC2HIC3 was not detrimental to its functional properties. Moreover, the reduction of the disulphide bonds in IEC2HIC3 resulted in decreased foam-forming properties (Chapter 7).

Chapters 2 and 3 showed that the amphipathic βCN peptides (i.e. βCN[f 1-105/107] and βCN[f 29-105/107]) had improved emulsion-forming properties (i.e. formed smaller particles) compared to the intact protein. The surface tension at the air-water interface was higher for the amphipathic βCN peptides than for the hydrophobic peptides and intact βCN (Chapter 2), and it is likely that this is also the case at the oil-water interface. A higher surface tension will give a larger Laplace pressure for particles of a certain size, which makes it more difficult to break up the particles into smaller ones (Walstra, 1993). Consequently, the surface tension cannot be the reason for the smaller particles formed by the amphipathic βCN

peptides. It can be hypothesised that, similar to intact βCN, the amphipathic βCN peptides form micelles in solution. However, the micellar interactions of the amphipathic peptides will be weaker than those of intact βCN, because they lack the hydrophobic C-terminal part of intact βCN. This could imply that during foam and emulsion formation the amphipathic peptides are better available for adsorption at the interface than the βCN molecules, resulting in the improved emulsion-forming properties of the amphipathic peptides. Homogenising at 4°C (when no βCN micelles are present) slightly decreased the emulsion-forming properties of intact βCN (Smulders, 1999) but it has to be noticed that the viscosity of the oil is higher at lower temperature making it more difficult to break up the droplets. Nevertheless, it is unlikely that the increased availability of the amphipathic peptides, due to the weaker micelles, are responsible for the improved emulsion-forming properties. Presumably, the improved emulsion-forming properties of the amphipathic βCN peptides βCN[f 1-105/107] and βCN[f 29-105/107] is due to the diminished coalescence during emulsion formation of these amphipathic peptides compared to that of βCN (Smulders et al., 1998).

Unlike proteins, peptides can desorb more easily from the interface (Smulders et al., 1998), which may (partly) explain the low foam- and emulsion-stability observed for the amphipathic βCN peptides βCN[f 1-105/107] and βCN[f 29-105/107]. Since the amphipathic βCN peptides are approximately half the size of intact βCN, theoretically twice as many molecules could adsorb at the interface. This would result in a layer with twice as much highly charged N-terminal ends present and consequently more electrostatic repulsion. However, the surface load found for these amphipathic peptides was lower than that of βCN and of the hydrophobic peptides (about 2.4 and 3.8 mg/m^2, respectively; Smulders et al., 1998; see also Chapter 4). This will probably imply similar to slightly increased electrostatic repulsion but decreased steric repulsion. The latter will probably cause the faster coalescence of the emulsions formed by the amphipathic peptides compared to those formed by intact βCN. Figure 2 depicts a possible conformation of βCN and of its derived amphipathic peptides on the interface. The large difference in emulsion

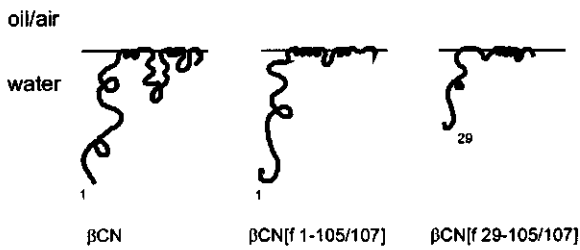

Figure 2 Assumed conformation of βCN and derived amphipathic peptides adsorbed at the oil/water or air/water interface (highly schematic and not to scale).

stability found for the two different amphipathic peptides is presumably caused by the difference in electrostatic repulsion: βCN[f 1-105/107] is more able to prevent coalescence (even in the presence of hydrophobic impurities) than is βCN[f 29-105/107], apparently because of the more highly charged N-terminus of the former (Chapter 3). This electro-steric repulsion becomes even more critical when the steric repulsion is less due to a low molecular size or to a low surface load. Preliminary experiments with partial and complete dephosphorylated βCN and βCN[f 1-105/107] showed that the surface load of the samples increased after dephosphorylation. However, dephosphorylation had no clear effect on the emulsion-forming and -stabilising properties (no further results shown). The decreased electrostatic repulsion (due to the removal of the phosphate groups) is probably compensated by the increased steric repulsion (due to the increased surface load), which explains the unchanged emulsion stability.

Hydrophobic peptides

The hydrophobic βCN peptides from the C-terminal half of βCN are poorly soluble at neutral pH, where they form highly aggregated emulsions and foams (Chapter 2). Since the hydrophobic peptides are poorly soluble at pH 6.7, the peptide solution contains solid peptide particles. These peptide particles adsorb strongly at the interface because of their high hydrophobicity but they do not cause sufficient electrostatic repulsion. The aggregation of the emulsions and foam might be due to strong, hydrophobic interactions between the peptides and peptide particles. Another explanation for the highly aggregated emulsions and foams is bridging flocculation due to the solid particles (Walstra, 1987).

At higher pH (pH 9.0) the hydrophobic βCN peptides are soluble, and it is remarkable that these peptides gave a (high) surface load, comparable to that of βCN (Chapter 4; Smulders et al., 1998). At this high pH they are able to form foam and emulsions with a reasonable stability against aggregation and coalescence, probably because they can produce enough repulsion due to the high surface load and charge, as shown by Smulders et al. (1998) for emulsions. Patel (1994) concluded that high solubility, high hydrophobicity, amphipathicity, flexibility (lack of secondary structure) and a strong tendency to form peptide-peptide interactions were factors in the superior foam properties of a casein peptide mixture. These factors were described to peptides originating from the C-terminal hydrophobic half of βCN (i.e. βCN[f 101-145], βCN[f 107-145] and βCN[f 107-135]) identified in the casein peptide mixture (Patel, 1994). These factors might also explain the high surface load found for the hydrophobic βCN peptides (Chapter 4), and consequently the good functional properties of these peptides at high pH.

SH/SS exchange during hydrolysis of β-lactoglobulin

The βLg peptides present in IEC2HIC3 (Chapter 7) contained mainly disulphide-linked peptides (with many newly formed disulphide bonds). Recently, a tryptic βLg peptide was assigned to the sequence βLg[f 102-124] -S-S- [f 149-162], with a newly formed disulphide bond (Maynard et al., 1998). The free thiol group at position 121 in βLg is known to initiate heat-induced aggregation by SH/SS exchange of the intact molecule (Hoffmann and Van Mil, 1997; Burova et al., 1998). Obviously, this SH/SS exchange is not only induced by (heat-)denaturation but also by hydrolysis (Chapter 7). Besides the free thiol group, the fragment βLg[f 142-162] is quite reactive in forming new disulphide bonds as well. The original disulphide bond between the residues 66 and 160 is on the outer surface, while the other disulphide bond (106-119) and the free thiol group (121) is buried in the interior of the native molecule (Papiz et al., 1986). No peptide bonds were split in the region between the Cys residues at positions 106, 119 and 121, because no Arg and Lys residues (specificity of plasmin) are present in that part of the βLg sequence. Therefore, the results obtained in Chapter 7 could not clarify whether the disulphide bond between 106 and 119 remains intact or that this bond also takes part in the reshuffling.

None of the βLg peptides identified in Chapter 7 consisted of more than two fragments linked together. Theoretically, larger aggregates were possible, since some identified peptide chains contained 2 or 3 cysteine residues. The reason that no larger fragments were found is probably the high amount of highly reactive thiol groups present, which very rapidly terminate the disulphide exchange reaction. In other words: reactive SH groups will quickly be "caught" by other reactive SH groups. This is in line with results published on the heat-induced aggregation of βLg, where the faster termination reaction at pH 8 compared to pH 6 is mentioned as an important factor for the formation of smaller aggregates at the higher pH (Hoffmann, 1997). So, although a lot of SH/SS exchange takes place during βLg hydrolysis, this seems not to result in large, covalently bound peptide aggregates, which is in agreement with literature (Otte et al., 1997b).

It has been shown that βLg hydrolysates are more efficient in gelation than is native βLg (Chen et al., 1994; Otte et al., 1997b). Although disulphide exchange reactions are thought to play an initiating role in the thermally induced aggregation of βLg, their role is considered to be minor compared with the other factors involved, such as hydrophobic and electrostatic interactions (Roefs and De Kruif; 1994; Sawyer et al., 1994). Otte et al. (1997b) showed that the non-covalent interactions were the main interacting forces in the aggregates formed during βLg hydrolysis with proteases from *Bacillus licheniformis*. In Chapter 6 it is also shown that peptides in the βLg hydrolysates can be linked by hydrophobic interactions. After βLg hydrolysis, more hydrophobic groups are exposed, which will presumably explain this hydrophobic aggregation, and also the more efficient gelation described (Chen et al., 1994; Otte et al., 1997b).

Secondary structure of β-casein (-peptides)

The results in Chapter 4 showed that βCN and its peptides had a low amount of ordered secondary structure in solution (presumably due to the high content of proline residues), which is in line with literature (Creamer et al.; 1981; Graham et al., 1984; Chaplin et al., 1988). It was also reported (Chaplin et al., 1988) that, despite the low amount of secondary structure in solution, some βCN peptides had α-helix forming ability when high amounts of trifuoroethanol (TFE) were added. The ability to form ordered secondary structure in solutions with TFE might be related to the ability to form ordered structures at the interface (Enser et al., 1990). The results presented in Chapter 4 showed that, despite the high content of proline residues, ordered structure is induced in βCN and its derived peptides after adsorption at hydrophobic teflon particles. The N-terminal amphipathic peptides (βCN[f 1-105/107] and βCN[f 29-105/107]) showed more structure induction than did the hydrophobic C-terminal peptides (βCN[f 106/108/114-209]) and the intact protein. The more amphipathic peptide (βCN[f 1-105/107)]) had a higher content of α-helix after adsorption than had the less amphipathic peptide (βCN[f 29-105/107)]) at the same surface load. Structure prediction showed that the segment 20-32 of βCN has helix-forming ability (Rost and Sander, 1993; 1994; Rost et al., 1994). The difference in amount of helix induction found for the two amphipathic peptides might be explained by the absence of the above-mentioned segment in the less amphipathic peptide. Dephosphorylation of intact βCN and βCN[f 1-105/107] increased the amount of α-helix induction upon adsorption (results not shown), which is in line with literature (Chaplin et al., 1988).

Several authors (Enser et al., 1990; Carey et al., 1994; Saito et al., 1995; Kang et al., 1996; Sheehan et al., 1998) have suggested a relationship between the presence of secondary structure in synthetic peptides in solution and biophysical properties. Especially a close correlation between the amphiphilic α-helical content (i.e. hydrophilic residues at one side of the helix and hydrophobic residues at the other side) and the emulsion-forming ability was found (Enser et al., 1990; Saito et al., 1995). In our studies on the functional properties of the βCN peptides (Chapter 2 and 3) the amphipathic peptides, having the highest amount of α-helix induction upon adsorption (see above), had improved emulsion-forming properties compared to the intact protein. This might suggest the earlier mentioned relationship with the secondary structure of synthetic peptides (Enser et al., 1990; Carey et al., 1994; Saito et al., 1995; Kang et al., 1996; Sheehan et al., 1998). However, the large differences found between the emulsion-stabilising properties of the amphipathic peptides and those of intact βCN do not point to the importance of structure induction but more to a relation with the surface load. Therefore, it was concluded that no clear relationship between the secondary structure induction of βCN peptides and their functional properties existed.

Concluding remarks

An important difference between βLg and βCN is their structure. βLg is a compact globular protein with two disulphide bonds and a free thiol group which can be described as a condensed ball. βCN is a flexible, random coil protein without cysteine residues which forms micelles above 4°C and above a critical concentration. Figure 3 schematically depicts the effects of βLg and βCN hydrolysis on their structure and thereby on the functional properties of the peptides.

The effects of enzymatic hydrolysis differ significantly between these proteins. The production of specific peptides and peptide fractions is more complicated for βLg than for βCN. This is mainly due to differences in primary structure, such as the distribution of charge and hydrophobicity, between the proteins. Considerable SH/SS exchange takes place during βLg/plasmin hydrolysis yielding an enormous amount of peptides. Due to this SH/SS exchange and to the homogenous distribution of charge and hydrophobicity, the βLg peptides are all rather alike. In contrast to this, βCN/plasmin hydrolysis yields peptides with distinct variability in hydrophobicity (and solubility) having clear differences in functionality.

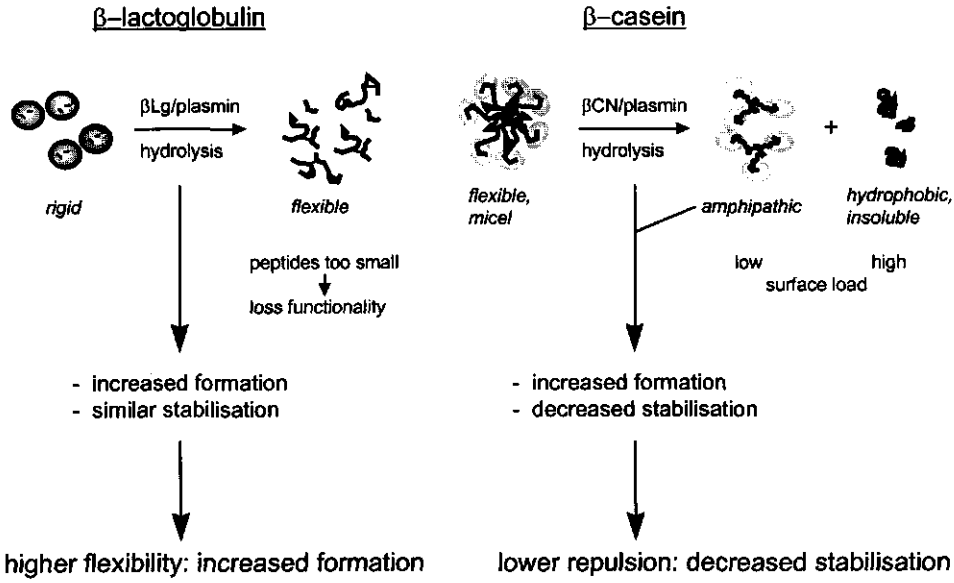

Figure 3. Summarising main effects of βLg/plasmin and βCN/plasmin hydrolysis on functionality; ⁄ disulphide bond; ◌ highly charged N-terminal end βCN(peptides); highly schematic.

When comparing the functionality of the intact proteins and their derived peptides the main conclusions are:

- the more flexible structure of the βLg peptides compared to that of the intact protein results in hydrolysates having increased foam- and emulsion-forming properties and similar stabilising properties;
- at acidic pH the functionality of the βCN peptides is superior to those of intact βCN, probably due to the increased solubility;
- at neutral pH the repulsion caused by the amphipathic βCN peptides is lower than that caused by intact βCN resulting in the decreased stabilising properties.

The approach of the study also enabled us to investigate structure-function relationships of accurately characterised peptides and peptide fractions. This gave the following main conclusions:

- the foam- and emulsion-forming properties of peptides can be superior to those of intact proteins, as long as the peptide structures have both charged and hydrophobic areas;
- the foam- and emulsion-stabilising properties of peptides depend on the amount of repulsion and is more critical for smaller molecules than for intact proteins; this repulsion can be caused by:
    - a strong amphipathicity of the peptide (for emulsions);
    - a high surface-load (for both foam and emulsions);
- no clear relationship existed between the secondary structure induction of βCN(-peptides) and the functional properties.

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Summary

The aim of this thesis was to elucidate the structure-function relationship for peptides derived from β-casein (βCN) and β-lactoglobulin (βLg) in regard to their foam- and emulsion-forming and -stabilising properties (further denoted functional properties). To this end, the proteins were modified by enzymatic hydrolysis, the hydrolysates were fractionated and characterised, and the functional properties of these peptides and peptide mixtures were determined. In this way, the structure-function relationship of βCN peptides and βLg peptides in relation to their functionality could be discussed at a molecular level. A general introduction to this subject is given in Chapter 1.

Chapter 2 describes the hydrolysis of bovine βCN by the action of plasmin. The hydrolysate was fractionated by selective precipitation and ultrafiltration, which resulted in several peptide fractions of which the peptide composition was monitored by reversed-phase HPLC. Poorly soluble, hydrophobic peptide fractions, containing peptides from the C-terminal half of the βCN sequence, had improved foam-forming and -stabilising properties compared to those of intact βCN, especially at pH 4.0. Soluble peptide fractions, containing a variety of peptides from the "middle" part of the βCN sequence in different proportions, possessed improved emulsion-forming properties at pH 6.7 compared to intact βCN, and showed large variations in emulsion stability. The fraction containing only the hydrophilic N-terminal part of βCN showed inferior foam, emulsion and surface-active properties, especially at pH 6.7. It is concluded that the differences in functionality found between the various peptide fractions may be attributed either to synergistic effects between peptides or to a specific functionality of some individual peptides.

In Chapter 3 the isolation and identification of peptides present in the βCN peptide fractions of Chapter 2 is described. An amphipathic peptide fraction was fractionated further by ion-exchange chromatography and subsequent hydrophobic-interaction chromatography, resulting in the components βCN[f 1-105/107] and βCN[f 29-105/107]. The latter peptides had poor emulsion-stabilising properties compared to the former ones. The stability of an emulsion formed with βCN[f 29-105/107] was also more sensitive to hydrophobic impurities than that of an emulsion formed with βCN[f 1-105/107]. It is concluded that the highly-charged N-terminal part is important for the emulsion-stabilising properties of these peptides.

Chapter 4 reports a study on the secondary structure of βCN and certain derived peptides (described in Chapters 2 and 3) both in solution and in the adsorbed state. The secondary structure was analysed by circular dichroism in order to investigate possible relationships with functional properties of the same protein/peptides (Chapters 2 and 3). A teflon/water interface was used as a model system for foam and emulsion interfaces. The main secondary structure element of all samples in solution was the unordered random coil, but upon adsorption ordered structures, especially α-helix, were induced. Apparently, both hydrophobic and

hydrophilic groups influenced the change of secondary structure induced at a hydrophobic interface. The results suggested that the hydrophobic C-terminal part of βCN accounted for the high maximum surface load on teflon, while the N-terminal part of βCN seemed to be responsible for the secondary structure induction upon adsorption. A relation between the maximum surface load and the foam-stabilising properties was found, but an influence of the secondary structure on the functionality was not observed in this system.

In Chapter 5 a method for preparing large quantities of native bovine βLg, suitable for research purposes, is described. The protein was isolated by combining a precipitation process and a diafiltration process. The yield of purified product from cheese whey protein fraction was approximately 60%. The chemical composition and the physicochemical properties of the purified βLg were determined. The results of the physicochemical determinations indicated that, using the method described in this Chapter, βLg of a high quality could be produced in a reproducible and relatively easy manner.

Chapter 6 describes the hydrolysis of βLg by the action of trypsin, plasmin and *Staphylococcus aureus* V8 protease (*S.aur.*V8) to the degrees of hydrolysis (DH) of 1, 2 and 4%. The peptide compositions of the several hydrolysates were determined by several chromatographic techniques. It was shown that the peptides formed were linked by hydrophobic interactions, by disulphide bonds, or were not linked at all. The hydrolysates showed similar or increased foam- and emulsion-forming properties in comparison with the intact protein. All emulsions formed had a similar stability, except for those formed with *S.aur.*V8, which had a poor stability. All foams formed were stable. Overall, the plasmin hydrolysate (DH4) contained a relatively high proportion of rather large peptides of which many were disulphide linked peptides. This fraction had the best functional properties at pH 6.7, compared to the other hydrolysates.

Chapter 7 describes the fractionation of the βLg/plasmin DH4 hydrolysate by ion-exchange chromatography and hydrophobic-interaction chromatography, and the identification of the βLg peptides. The βLg peptide fraction consisting of smaller peptides (mostly < 2 kDa) had poor foam- and emulsion-forming and -stabilising properties. Many of the peptides present in the peptide fraction having good functional properties, were larger peptides having many hydrophobic groups, and of which many were disulphide-linked fragments. The peptides formed during βLg/plasmin hydrolysis were (1) peptides composed of a single amino acid chain lacking a cysteine residue, (2) peptides composed of a single amino acid chain containing intramolecular disulphide bonds and (3) peptides composed of 2 amino acid chains linked by an intermolecular disulphide bond. It appeared that significant SH/SS-exchange had taken place during hydrolysis.

In Chapter 8 the main results of the thesis are briefly discussed. Summarising, it can be concluded that the production of specific peptides and peptide fractions is more complicated for βLg than for βCN, mainly because of the differences in primary structure (such as the distribution of charge and hydrophobicity) between the proteins. The foam- and emulsion-forming properties of

peptides can be superior to those of intact proteins, as long as they have both charged and hydrophobic areas. The foam- and emulsion-stabilising properties of peptides depend strongly on the amount of repulsion. No clear relationship was found between the secondary structure of βCN(-peptides) and the functional properties.

Samenvatting

Het onderzoek dat in dit proefschrift is beschreven had als doel de structuur-functierelatie te onderzoeken van peptiden afkomstig van β-caseïne (βCN) en β-lactoglobuline (βLg) met betrekking tot hun schuim- en emulsievormende en -stabiliserende eigenschappen (ook wel functionele eigenschappen genoemd). Hiertoe zijn de twee genoemde eiwitten (beide melkeiwitten) enzymatisch gehydrolyseerd (oftewel in kleinere stukken geknipt), waarna de hydrolysaten gefractioneerd en gekarakteriseerd zijn. Vervolgens zijn de schuim- en emulsie-eigenschappen van de verschillende peptiden en peptidemengsels bepaald. Op deze manier kon de structuur-functierelatie op een moleculair niveau bediscussieerd worden. Een algemene inleiding over dit onderwerp staat in Hoofdstuk 1.

In Hoofdstuk 2 is de hydrolyse van βCN met het enzym plasmine beschreven. Het hydrolysaat is gefractioneerd door middel van selectieve precipitatie en ultrafiltratie. Hierdoor zijn verschillende fracties verkregen waarvan de peptidesamenstelling met behulp van reversed-phase HPLC (een techniek die voornamelijk scheidt op hydrofobiciteit oftewel watermijdende eigenschappen) geanalyseerd is. Slecht oplosbare, hydrofobe peptiden (afkomstig van het C-terminale deel van het eiwitmolecuul) hadden, vooral bij pH 4, verbeterde schuimvormende en -stabiliserende eigenschappen vergeleken met het intacte eiwit. De oplosbare peptidefracties, die een mengsel van peptiden van het middelste deel van het βCN-molecuul bevatten, hadden verbeterde emulsievormende eigenschappen bij pH 6.7 (de pH van melk) ten opzichte van βCN. Deze fracties hadden sterk uiteenlopende emulsiestabiliserende eigenschappen. De fractie met alleen het hydrofiele (oftewel waterminnende) N-terminale deel van het βCN-molecuul had slechte functionele eigenschappen. De conclusie van het onderzoek beschreven in dit hoofdstuk is dat de verschillen tussen de diverse peptidefracties veroorzaakt zijn door een synergistische (oftewel elkaar versterkende) werking tussen de aanwezige peptiden, of door een specifieke functionaliteit van enkele individuele peptiden.

In Hoofdstuk 3 is de isolatie en identificatie beschreven van de peptiden aanwezig in de fracties beschreven in Hoofdstuk 2. Een amfipatische ("met een strikte scheiding tussen geladen en hydrofobe groepen") peptidefractie is verder gefractioneerd door middel van ionenwisselingschromatografie (een techniek waarbij vooral op lading gescheiden wordt) en vervolgens hydrofobe-interactie-chromatografie (een techniek waarbij vooral op hydrofobiciteit gescheiden wordt). Hierdoor zijn de peptidefracties βCN[f 1-105/107] en βCN[f 29-105/107] verkregen. Deze laatste peptiden hadden slechte emulsiestabiliserende eigenschappen vergeleken met de eerste groep peptiden. De stabiliteit van een emulsie gevormd met βCN[f 29-105/107] was ook gevoeliger voor vervuiling met hydrofobe stoffen. Hieruit is geconcludeerd dat het sterk geladen N-terminale deel (1-28) van de

peptiden erg belangrijk is voor de emulsiestabiliserende eigenschappen van de peptiden.

In Hoofdstuk 4 is de studie naar de secundaire structuur (oftewel lokale vouwingspatronen) van βCN en enkele βCN-peptiden in oplossing en in geadsorbeerde toestand beschreven. De secundaire structuur (bepaald met behulp van de techniek circulair dichroïsme) is geanalyseerd om een mogelijk verband tussen deze secundaire structuur en de eerder bepaalde functionele eigenschappen (Hoofdstuk 2 en 3) te onderzoeken. Een teflon/water oppervlak is gebruikt als modelsysteem voor de oppervlakken die in schuim en emulsies aanwezig zijn. Het belangrijkste secundaire-structuurelement aanwezig in oplossing was de random coil (oftewel ongeordende) structuur, maar na adsorptie werd geordende secundaire structuur (voornamelijk α-helix) geïnduceerd. Zowel hydrofobe als hydrofiele delen van het eiwit bleken van belang te zijn voor deze structuurinductie. De resultaten suggereerden dat het hydrofobe C-terminale deel van βCN verantwoordelijk is voor de hoge belading van βCN op het teflon, terwijl het N-terminale deel van βCN verantwoordelijk lijkt te zijn voor de structuurinductie na adsorptie aan het oppervlak. Er is een relatie gevonden tussen de maximale belading van het βCN(-peptide) op het teflon en de schuimstabiliserende eigenschappen, maar er lijkt geen verband te bestaan tussen de secundaire structuur en de functionele eigenschappen.

In Hoofdstuk 5 is een zuiveringsmethode voor grote hoeveelheden βLg (geschikt voor onderzoek) beschreven. Het eiwit is geïsoleerd door een combinatie van selectieve precipitatie en ultrafiltratie. De opbrengst van het gezuiverde eiwit bij zuivering uit een kaaswei-eiwitfractie was ongeveer 60%. De chemische samenstelling en de fysisch-chemische eigenschappen van het gezuiverde βLg zijn bepaald. De resultaten toonden aan dat met de beschreven methode βLg van een hoge kwaliteit verkregen kan worden op een reproduceerbare en relatief eenvoudige manier.

In Hoofdstuk 6 is de hydrolyse van βLg door de enzymen trypsine, plasmine en *Staphylococcus aureus* V8 protease (*S.aur.*V8) tot de hydrolysegraad (DH) van 1, 2 en 4% beschreven. De peptidesamenstelling is geanalyseerd met verschillende chomatografische methoden. Er is aangetoond dat de peptiden in die hydrolysaten of niet met elkaar verbonden zijn, of door hydrofobe interacties en/of door zwavelbruggen (bindingen tussen de zwavelgroepen van cysteïneresiduen) met elkaar verbonden zijn. De schuim- en emulsievormende eigenschappen van alle hydrolysaten waren gelijk aan of beter dan die van het intacte βLg. Alle emulsies hadden een vergelijkbare stabiliteit, behalve de emulsies gevormd met *S.aur.*V8 hydrolysaten, die een slechte stabiliteit vertoonden. Alle hydrolysaten vormden stabiel schuim (evenals βLg). In het algemeen waren in het plasminehydrolysaat met een DH van 4% (DH4) relatief de meeste grote peptiden aanwezig, van welke vele verbonden waren door zwavelbruggen. Dit hydrolysaat had, in vergelijking met de andere hydrolysaten, de beste functionele eigenschappen bij pH 6.7.

In Hoofdstuk 7 is de fractionering van het βLg/plasminehydrolysaat DH4 (beschreven in Hoofdstuk 6) door middel van ionenwisselingschromatografie en

hydrofobe-interactiechromatografie beschreven, evenals de identificatie van de aanwezige βLg peptiden. De fractie met slechts kleine peptiden (massa voornamelijk kleiner dan 2000 Da) had zeer slechte functionele eigenschappen. In de fractie die goede functionele eigenschappen vertoonde, waren grote peptiden aanwezig, waarvan vele peptiden bestonden uit fragmenten die met zwavelbruggen verbonden waren. De peptiden gevormd tijdens de βLg hydrolyse met plasmine waren (1) peptiden bestaande uit een enkele aminozuurketen zonder een cysteïneresidu; (2) peptiden bestaande uit een enkel aminozuurketen met 2 of 4 cysteïneresiduen en 1 of 2 zogenoemde intramoleculaire zwavelbruggen; of (3) peptiden samengesteld uit 2 aminozuurketens met elk 1 of 3 cysteïneresiduen en met elkaar verbonden door minstens 1 zogenoemde intermoleculaire zwavelbrug. Uit het onderzoek in dit hoofdstuk is gebleken dat tijdens βLg/plasminehydrolyse veel uitwisseling van zwavelbruggen heeft plaatsgevonden.

In Hoofdstuk 8 zijn de belangrijkste resultaten van het onderzoek bediscussieerd. Samenvattend kan geconcludeerd worden dat de productie van specifieke peptiden(fracties) gecompliceerder is in het geval van βLg dan in het geval van βCN. Dit komt voornamelijk door het verschil in primaire structuur tussen de eiwitten, zoals bijvoorbeeld door de verdeling van geladen en hydrofobe groepen over het molecuul. De aan- dan wel afwezigheid van zwavelbruggen kan ook een rol spelen. De schuim- en emulsievormende eigenschappen van peptiden kunnen beter zijn dan die van de intacte eiwitten, maar dan moeten de peptiden zowel geladen als hydrofobe groepen bezitten. De schuim- en emulsiestabiliserende eigenschappen van de peptiden worden bepaald door de mate waarin deze peptiden in staat zijn repulsie (afstoting) tussen deeltjes (schuimbellen/emulsiedruppels) op te wekken. Er is geen verband gevonden tussen de secundaire structuur van βCN(-peptiden) en hun functionele eigenschappen.

Nawoord

Het is af!! En toch nog sneller dan verwacht! Ik wil op deze plek enkele mensen noemen die ervoor gezorgd hebben dat ik gedurende mijn AIO-periode veel heb geleerd en met plezier gewerkt heb.

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Ik wil hier ook graag de overige bij het IOP-project betrokkenen noemen. Mijn collega-AIO's Ine Smulders en Arne van Kalsbeek: bedankt voor de goede samenwerking en de onderlinge besprekingen. Ik hoop dat jullie mij niet blijven herinneren als iemand die enorm krenterig was als het ging om het leveren van gezuiverde peptiden..... De bijdragen aan het project van Prof. A. Prins en Prof. Pieter Walstra heb ik zeer gewaardeerd. Pieter, bedankt voor je nuttige commentaar op mijn verslagen en manuscripten en voor de antwoorden op tussentijdse vragen. Verder heb ik de bijdragen aan de halfjaarlijkse vergaderingen van alle leden van de begeleidingscommissie van het IOP-IE gewaardeerd.

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Curriculum Vitae

Petra Caessens is op 15 juli 1969 geboren in Geleen. In 1987 heeft zij het VWO diploma aan de Scholengemeenschap St. Michiel te Geleen behaald waarna zij begon met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. In augustus 1993 studeerde zij af met als afstudeervakken Zuivelkunde en Toxicologie en met stages bij het Zuivelbedrijf "De Vereeniging" te Limmen en bij het Food Research Institute te Werribee, Australië.

Van februari 1994 tot augustus 1998 werkte ze als assistent in opleiding (AIO) bij de Leerstoelgroep Levensmiddelenchemie van het Departement Levensmiddelentechnologie en Voedingswetenschappen aan de Landbouw-universiteit Wageningen. Een gedeelte van het onderzoek heeft plaatsgevonden bij de afdeling Biofysische Chemie (later bij de afdeling Product Technology) van NIZO food research te Ede. Het onderzoek uitgevoerd in deze periode staat beschreven in dit proefschrift.

Vanaf januari 1999 is zij werkzaam als onderzoeker bij DMV-International te Veghel.

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